

2)

THE MONITORING AND EVALUATION **OF A SOLID SUBSTRATE SUBMERGED CULTURE** **FERMENTATION**


By
Max James Kennedy
BE (Chemical) with First Class Honours, 1983
The University of Auckland
NEW ZEALAND


Submitted to the Department of Chemical Engineering,
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy,

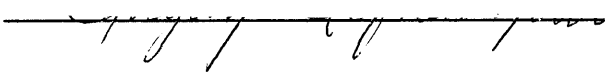
at the
Massachusetts Institute of Technology,
June, 1990

• Max James Kennedy, 1990, all rights reserved.

The author hereby grants to MIT permission to reproduce and
distribute copies of this thesis in whole or in part.

Signature of Author _____
 Max James Kennedy
27 June, 1990

Certified by _____
 Daniel I. C. Wang
Thesis Supervisor

Certified by _____
 Gregory N. Stephanopoulos
Thesis Supervisor

Accepted by _____
William M. Deen
Chairman, Departmental Committee on Graduate Students

MASSACHUSETTS INSTITUTE
OF TECHNOLOGY

OCT 04 1990

LIBRARIES

ABSTRACT

A novel light scatter sensor was developed to estimate cell concentration in the presence of solid substrate. The light scatter technique is based on two observations. First, that the light scatter from cells is a near linear function of cell concentration. Second, that invariant regions are present in the light scatter spectrum of cell/solid substrate mixtures. Invariant regions are regions of the light scatter spectrum in which the light scatter reading is a function of cell concentration but is independent of solid substrate concentration.

The light scatter technique was validated by comparing the technique to the DNA and carbon dioxide evolution techniques during a series of fermentations. The model system used was the production of proteases by *Bacillus subtilis* var *sakainensis* ATCC 21394 growing on fishmeal as the sole nitrogen source. Batch and fed-batch fermentations, and a consideration of protease regulation indicates that the addition of solid substrate during the fermentation could improve fermentation performance.

ACKNOWLEDGEMENTS

Firstly I would like to acknowledge and thank my advisors, Dr Daniel Wang and Dr. Greg Stephanopoulos. Thank you Dr Wang for your encouragement and support, for guiding me, for showing me how to take a calculated risk and for always setting a professional standard for me to aim at. Your words of wisdom will remain with me. In New Zealand we would say that some of your *mana* has rubbed off on me. Thank you Dr Stephanopoulos for the many long discussions, and your enthusiasm and interest in my work. Thanks also to my thesis committee, Dr C.L. Cooney and Dr A.L. Demain. Your constructive criticism along the journey was appreciated.

Next I would like to thank my colleagues. The old timers, Dave Robinson, Jim McMillan, Sydar Wang, Greg O'Connor, Hong Woo Park and Vijay Yabanavar who initiated me into the lab and passed on all the mysteries, tricks and traps of doing a fermentation. Thanks to Beth Junker for showing me the ropes of using the often temperamental fluorometer. A special thanks to Anna Hagen and Steve Lee for sharing the everyday life in the lab. Whenever I needed a boost, a new angle or a guinea pig to listen to a new idea Steve was always there for me. Thanks also to those who followed behind, Jeff Cleland, Cliff Rutt and Eric Scharin. Peggy Foster also deserves a special mention and thanks, she provided me with a ready smile and had a keen interest in all the students welfare.

This work could not have been completed without the help of Dr. Munna Thakur. I shall miss Munna's contagious optimism and enthusiasm. Thanks for putting in all those hours Munna. I learned a lot from you. Also thanks to UROP Richard Seitz and REU

student Mike Powell who have shared the battleground and taught me managerial skills.

Thanks to those who provided skills that I lacked, at just the right time. Thanks to Seujeung Park for optimizing the model, Joe Hong from Ohaus Corporation for getting the multiplexer up and running and James Baker for getting my data from one computer to another.

I would like to thank those who provided the inspiration to undertake my PhD, especially Dr P. Munro and Dr D. Bell who first sowed the seeds for this adventure. This PhD would not have been possible if it wasn't for the support (financial and otherwise) of the New Zealand Department of Scientific and Industrial Research, especially the Industrial Processing Division and the New Zealand National Research Advisory Council. Your support is greatly appreciated. The author also wishes to acknowledge the financial aid of the M.I.T. Biotechnology Process Engineering Center through the United States National Science Foundation - ERC Initiative under cooperative agreement CDR-88-03014. This financial support was invaluable.

To my family who nourished me and made me what I am today, especially my mother and father and also to my three big sisters, Irene, Christene and Maureen, thanks. A special thanks also to Max, Blen, Dennis and Terence for making a happy family. Your care and concern and all the correspondence about what was happening back in New Zealand really helped.

Finally I would like to honor and thank my wife Maria and my daughter Emma for their love, support and caring. They truly carried the load with me and this PhD partly belongs to them. We made a great team, thanks doesn't seem enough.

DEDICATION

This thesis is dedicated to all those who have shown me their love,
especially Maria, Emma, my mother and father,
and Irene, Christene and Maureen.

TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	ii
DEDICATION	iv
TABLE OF CONTENTS	v
LIST OF FIGURES	ix
LIST OF TABLES	xv
 1) INTRODUCTION	 1
1.1) Background	1
1.2) Motivation	2
1.3) Problem Statement	6
1.4) Objectives	6
1.5) Model System	7
 2) LITERATURE SURVEY	 9
2.1) <i>Bacillus subtilis</i>	9
2.1.1) <i>Bacillus subtilis</i> var <i>sakainensis</i> ATCC 21394	10
2.2) Solid Substrates Used in Fermentation Medium	10
2.2.1) Fishmeal	12
2.3) Protease Production	13
2.3.1) Proteases: The Basics	13
2.3.2) The Development of Protease Production	14
2.3.3) Protease Production Technology	17
2.3.4) Notes on Reading the Protease Literature	23
2.4) Measuring Cell Concentration in the Presence of Solid Substrate	23
2.5) The Application of Light Scatter Techniques to Monitor Fermentation ..	33

3) MATERIALS AND METHODS	39
3.1) Equipment	39
3.1.1) Fermentor	39
3.1.2) Data Acquisition and Control Systems	41
3.1.3) Fluorometer	44
3.2) Assays and Techniques	52
3.2.1) DNA Assay to Estimate Cell Concentration	52
3.2.2) Carbon Dioxide Evolution Technique to Estimate Cell Concentration	69
3.2.3) Fishmeal Assay	74
3.2.4) Glucose, Acetate and Acetoin Assays	76
3.2.5) Protease Assay	81
3.2.6) Light Scatter Spectrum Measurement Technique	86
3.3) Equipment Setup and Control Strategies	89
3.3.1) Equipment Setup	89
3.3.2) Glucose Control Strategy	95
3.3.3) Dissolved Oxygen Control Strategy	102
3.3.4) pH Control	104
3.3.5) Aeration Control	104
3.3.6) Agitation Control	104
3.3.7) Foam Control	105
3.3.8) Temperature Control	105
3.4) Culture Handling and Media	106
3.4.1) Culture Storage	106
3.4.2) Media	107
3.4.3) Inoculum Development and Fermentor Preparation	110
4) RESULTS AND DISCUSSION	115
4.1) The light Scatter Spectra of Cells and Solids	115
4.2) The Effect of Concentration on the Light Scatter Spectrum	118

4.3) The Effect of Probe Geometry	126
4.4) The Light Scatter Spectrum of Mixtures of Cells and Solids	129
4.5) The Effect of Particle Size on the Light Scatter Spectrum	156
4.6) Estimation of Cell Concentration in the Presence of Solid Substrate using Light Scatter Data	160
4.7) Estimation of Low Cell Concentrations	173
4.8) A Model Describing the Light Scatter Spectrum of Cell and Solid Mixtures	177
4.9) Evaluation of Fermentation Data	186
5) CONCLUSIONS	198
6) SUGGESTIONS FOR FUTURE WORK	200
7) REFERENCES	206
Appendix A: FORTRAN Data Acquisition and Control Program.	A1
Appendix B: Paragon Data Acquisition and Control Program.	B1
Appendix C: Signal Conditioning Unit for the PC-Based Data Acquisition and Control System.	C1
Appendix D: Fluorescence Spectroscopy Version 1.1 program used to Control the Fluorometer during Light Scatter Experiments.	D1
Appendix E: A Model of a Solid Substrate Fermentation	E1
E.1) Introduction and Objectives of the Modeling Studies	E1
E.2) Literature Survey	E1
E.3) Overview and Structure of the Model	E9
E.4) Balance on Dissolved Fishmeal	E12
E.5) Balance on Cells	E17
E.6) Balance on Glucose	E21

E.7) Balance on Fishmeal	E25
E.8) Balance on Proteases	E33
E.9) Fit of the Model to the Batch and Fed-Batch Fermentations	E40
E.10) Extension of the Model and a Control Experiment	E49
E.11) Summary of the Model	E72
E.12) The Limitations to Alkaline Protease Production	E74
E.13) Strengths and Areas for Improvement of the Model	E74
Appendix F: BASIC Program used to Generate Model Fermentation Profiles.	F1
Appendix G: FORTRAN Program used to Optimize the Solid Substrate Model. . . .	G1
Appendix H: FORTRAN Program used to Determine the Fishmeal Feed Profile After 12 Hours in the Control Experiment.	H1
Appendix I: FORTRAN Program used to Estimate the PID Control Parameters in the Dissolved Fishmeal Control Strategy.	I1

Biographical Sketch

LIST OF FIGURES

Figure 1:	The BioFlo II fermentor and LB-60 scale.	40
Figure 2:	The PC-based control system.	42
Figure 3:	The PC-based control system signal conditioning board.	43
Figure 4:	Schematic diagram of the fluorometer and control system.	46
Figure 5:	Overall view of the fluorometer and Apple PC control system.	47
Figure 6:	The radiometers and arc lamp supply.	48
Figure 7:	Light train of the fluorometer, including the xenon arc lamp, ozone eater, photomultipliers and fiber optics.	49
Figure 8:	Side view of the front face fiber optic probe showing the excitation and emission fiber optic lines.	50
Figure 9:	End view of the front face fiber optic probe showing the excitation and emission fiber optic lines.	51
Figure 10:	The excitation and emission spectra of DAPI/calf thymus DNA under the conditions used in this study.	57
Figure 11:	The effect of temperature on the DAPI/calf thymus DNA fluorescent response.	58
Figure 12:	The effect of pH on the DAPI/calf thymus DNA fluorescent response.	59
Figure 13:	The effect of fishmeal addition on the DAPI/ <i>Bacillus subtilis</i> DNA fluorescent response.	60
Figure 14:	Calibration curve of fluorescence versus <i>Bacillus subtilis</i> concentration. Data from 3 fermentations.	61
Figure 15:	Calibration curve of fluorescence versus calf thymus DNA concentration.	62
Figure 16:	The effect of growth rate and spore formation activities on the DNA content of <i>Bacillus subtilis</i>	67
Figure 17:	A comparison of the DNA assay and dry cell weight techniques to estimate cell concentration during the fermentation of <i>Bacillus subtilis</i> on nutrient broth.	68

Figure 18:	The correlation of dry cell weight accumulated with total carbon dioxide evolved per liter. Data from six fermentations.	71
Figure 19:	The integrator program used to calculate HPLC results.	78
Figure 20:	Calibration curves for glucose, acetate and acetoin.	79
Figure 21:	A HPLC spectrum obtained from the fermentation of <i>Bacillus subtilis</i> on fishmeal.	80
Figure 22:	The wrapped sample assembly used to measure the light scatter spectrum	88
Figure 23:	A schematic diagram of the experimental setup used in this study. M signifies that data was manually entered.	91
Figure 24:	The overall experimental setup showing the fermentor, feed vessels, multiplexer, mass flow controller and Opto 22 signal conditioning unit. .	92
Figure 25:	Detail showing the glucose and NaOH feed vessels and balances.	93
Figure 26:	Detail showing the multiplexer and a mass flow controller.	94
Figure 27:	The calculation of K_A for fishmeal and nutrient broth as nitrogen sources	99
Figure 28:	Prediction of the glucose concentration in a nutrient broth fermentation using the K_A value calculated from the same data.	100
Figure 29:	The prediction of glucose concentration in a fishmeal fermentation using the K_A value calculated from the same data.	101
Figure 30:	Spores of <i>Bacillus subtilis</i> var <i>sakainensis</i> ATCC 21394 taken from a sporulation agar plate.	112
Figure 31:	Chains of <i>Bacillus subtilis</i> var <i>sakainensis</i> ATCC 21394 growing on fishmeal near the beginning of the fermentation. Taken under oil at 1000x magnification.	113
Figure 32:	Single cells of <i>Bacillus subtilis</i> var <i>sakainensis</i> ATCC 21394 growing on fishmeal near the end of the fermentation. Taken under oil at 1000x magnification.	114
Figure 33:	The light scatter spectra of <i>Saccharomyces cerevisiae</i> , <i>Bacillus subtilis</i> and <i>Escherichia coli</i>	116
Figure 34:	The light scatter spectra of fermentation substrates. Abbreviations: PO	

	(peanut oil), CS (corn starch), CSM (cottonseed meal), SBM (soybean meal), FM (fishmeal).	117
Figure 35:	The effect of cell concentration on the light scatter spectrum of <i>Saccharomyces cerevisiae</i>	119
Figure 36:	The effect of cell concentration on the light scatter spectrum of <i>Bacillus subtilis</i>	120
Figure 37:	The effect of cell concentration on the light scatter spectrum of <i>Escherichia coli</i>	121
Figure 38:	The response of integrated light scatter (470-480 nm) to cell concentration.	122
Figure 39:	The effect of cell concentration on the integrated light scatter (580-590 nm) of <i>Bacillus subtilis</i> up to 50 g/l.	123
Figure 40:	The effect of concentration on the light scatter spectrum of fishmeal. .	124
Figure 41:	The effect of concentration on the light scatter spectrum of cottonseed meal.	125
Figure 42:	Front face and right angle probe geometries.	127
Figure 43:	The light scatter at 540 nm as a function of <i>Bacillus subtilis</i> concentration obtained using a right angle probe.	128
Figure 44:	An invariant region in the light scatter spectra of mixtures of <i>Bacillus subtilis</i> and fishmeal at a cell concentration of 2.8 g/l.	134
Figure 45:	An invariant region in the light scatter spectra of mixtures of <i>Bacillus subtilis</i> and fishmeal at a cell concentration of 6.0 g/l.	135
Figure 46:	An invariant region in the light scatter spectra of mixtures of <i>Bacillus subtilis</i> and fishmeal at a cell concentration of 13.5 g/l.	136
Figure 47:	An invariant region in the light scatter spectra of mixtures of <i>Bacillus subtilis</i> and soybean meal (180-300 microns) at a cell concentration of 1.8 g/l.	137
Figure 48:	An invariant region in the light scatter spectra of mixtures of <i>Bacillus subtilis</i> and soybean meal (180-300 microns) at a cell concentration of 8.9 g/l.	138
Figure 49:	An invariant region in the light scatter spectra of mixtures of <i>Bacillus subtilis</i>	

	and soybean meal (180-300 microns) at a cell concentration of 17.2 g/l.	139
Figure 50:	An invariant region in the light scatter spectra of mixtures of <i>Saccharomyces cerevisiae</i> and fishmeal at a cell concentration of 5.5 g/l.	140
Figure 51:	An invariant region in the light scatter spectra of mixtures of <i>Saccharomyces cerevisiae</i> and fishmeal at a cell concentration of 11.0 g/l.	141
Figure 52:	An invariant region in the light scatter spectra of mixtures of <i>Saccharomyces cerevisiae</i> and fishmeal at a cell concentration of 22.7 g/l.	142
Figure 53:	An invariant region in the light scatter spectra of mixtures of <i>Escherichia coli</i> and fishmeal at a cell concentration of 5.4 g/l.	143
Figure 54:	An invariant region in the light scatter spectra of mixtures of <i>Escherichia coli</i> and fishmeal at a cell concentration of 10.1 g/l.	144
Figure 55:	An invariant region in the light scatter spectra of mixtures of <i>Escherichia coli</i> and fishmeal at a cell concentration of 14.4 g/l.	145
Figure 56:	An invariant region in the light scatter spectra of mixtures of <i>Saccharomyces cerevisiae</i> , cottonseed meal (CSM) and fishmeal (FM) at a cell concentration of 26.1 g/l.	146
Figure 57:	The location of the invariant region as a function of cell concentration for <i>Bacillus subtilis</i> and fishmeal.	147
Figure 58:	The location of the invariant region as a function of cell concentration for <i>Bacillus subtilis</i> and soybean meal..	148
Figure 59:	The location of the invariant region as a function of cell concentration for <i>Saccharomyces cerevisiae</i> and fishmeal.	149
Figure 60:	The location of the invariant region as a function of cell concentration for <i>Escherichia coli</i> and fishmeal.	150
Figure 61:	Center of the invariant region as a function of cell concentration for various cell/solid systems. Abbreviations: BS (<i>Bacillus subtilis</i>), EC (<i>Escherichia coli</i>), SC (<i>Saccharomyces cerevisiae</i>), FM (fishmeal) and SBM (soybean meal).	151
Figure 62:	The light scatter spectrum of <i>Bacillus subtilis</i> and cottonseed meal (180-300 microns). Note the absence of the invariant region.	152

Figure 63:	The light scatter spectrum of <i>Bacillus subtilis</i> and corn starch. Note the absence of the invariant region.	153
Figure 64:	An invariant region in the light scatter spectra of mixtures of <i>Bacillus subtilis</i> and fishmeal, at high fishmeal concentrations.	154
Figure 65:	No invariant regions were observed between <i>Bacillus subtilis</i> (3.7 g/l) and fishmeal using the right angle probe.	155
Figure 66:	The effect of solid particle size on the light scatter spectrum of <i>Saccharomyces cerevisiae</i> and soybean meal.	157
Figure 67:	The effect of solid particle size on the light scatter spectrum of <i>Bacillus subtilis</i> and soybean meal.	158
Figure 68:	The effect of solid particle size on the light scatter spectrum of <i>Bacillus subtilis</i> and fishmeal.	159
Figure 69:	The methodology used to calculate cell concentration from light scatter data.	166
Figure 70:	The graphical method of estimating cell concentration.	168
Figure 71:	The light scatter spectra obtained during a fermentation. The third dimension represents the time during the fermentation.	169
Figure 72:	Comparison of cell concentration predictions from the light scatter technique with those from the DNA technique during the growth of <i>Bacillus subtilis</i> on fishmeal.	170
Figure 73:	Comparison of cell concentration predictions from the light scatter technique with those from the DNA and carbon dioxide evolution techniques during the growth of <i>Bacillus subtilis</i> on fishmeal.	171
Figure 74:	Comparison of cell concentration predictions from the light scatter technique with those from the DNA and carbon dioxide evolution techniques during the fed-batch fermentation of <i>Bacillus subtilis</i> on fishmeal.	172
Figure 75:	The cell concentration obtained by correcting the light scatter data, compared with the DNA technique.	175
Figure 76:	The cell concentration obtained by correcting the light scatter data, compared	

	with DNA and carbon dioxide evolution techniques.	176
Figure 77:	Schematic diagram of the light scatter model.	181
Figure 78:	The goodness of fit of the model to the light scatter data.	182
Figure 79:	The use of the model to predict the light scatter spectrum. Data at low cell concentration.	183
Figure 80:	The use of the model to predict the light scatter spectrum. Data at high cell concentration.	184
Figure 81:	The solid absorbance index as a function of wavelength.	185
Figure 82:	The cell concentration profile obtained during the batch fermentation of fishmeal by <i>Bacillus subtilis</i>	192
Figure 83:	The fishmeal concentration profile obtained during the batch fermentation of fishmeal by <i>Bacillus subtilis</i>	193
Figure 84:	The glucose concentration profile obtained during the batch fermentation of fishmeal by <i>Bacillus subtilis</i>	194
Figure 85:	The alkaline protease activity profile obtained during the batch fermentation of fishmeal by <i>Bacillus subtilis</i>	195
Figure 86:	The glucose concentration profiles in the batch and fed-batch fermentations listed in table 6.	196
Figure 87:	The glucose concentrations obtained in the fed-batch fermentations (base case and the overfeeding experiment) shown in table 7.	197

LIST OF TABLES

Table 1:	Relative strength of toxic products and substrate on the inhibition of the growth of <i>Bacillus polymyxa</i> (data from Mas et al., 1988).	22
Table 2:	The composition of the DAPI/Buffer solution used in this study.	54
Table 3:	The linear slope between 4-20 g/l cells of the location of the invariant region as a function of cell concentration.	130
Table 4:	An example calculation taken from fermentation data of the growth of <i>Bacillus subtilis</i> on fishmeal.	167
Table 5:	The comparison of batch fishmeal fermentations.	189
Table 6:	Comparison of a batch with a fed-batch fishmeal fermentation.	190
Table 7:	The comparison of a base case with an overfeeding experiment in the fed-batch fermentation of fishmeal.	191

1) INTRODUCTION

1.1) Background

Historically the introduction of novel sensors to measure new parameters during fermentation have led to quantum leaps in our understanding of fermentation processes. For example, significant developments occurred after the introduction of the dissolved oxygen probe and the mass spectrometer for measuring off-gas composition. It is often the case that new developments in fermentation technology are sensor-limited. The problem is not lack of imagination in exploiting new information but obtaining the new information in the first place.

This need for new information has led to the development and application of many sophisticated techniques to estimate the needed variables. For example, the application of the coulter counter or nuclear magnetic resonance techniques to fermentation. Many of these complicated techniques have not been embraced by industry, but are restricted to research laboratories. There are three reasons for this. First, most of these techniques are off-line techniques which mean they are of limited use in controlling the fermentation in real time. Secondly most of the techniques do not work under harsh industrial conditions. For example the solid substrate used in many fermentation media interferes with most techniques. Thirdly, these eloquent techniques are very costly and require skilled personnel to operate the systems.

This lack of applicability to industry has meant that much of the promising research occurring in the laboratory stays just there, in the laboratory. This thesis aims in a small way to attempt to bridge the gap between the laboratory and industry. A novel sensing

technique was designed to measure the most fundamental variable, cell concentration, under conditions similar to those in industry. Once this was developed, we looked at the question "what biochemical engineering principles can we apply, based on this measurement, to try to improve the fermentation?"

1.2) Motivation

Knowing the cell mass concentration during a fermentation is crucial. Agar, 1985 states that "knowledge of the amount of biomass in a system and its increase with time, the growth rate, is fundamental to any work in biotechnology". Küenzi, 1978 states that "if one intends to influence the course of a fermentation while it is in progress, data on growth should be available immediately". Testifying to the importance of cell concentration measurement Harris and Kell, 1985 lists 24 reviews on biomass determination between 1969 and 1985.

Cell mass concentration or growth rate data are used in the following manner:

1) To compare fermentation performance by calculating specific productivity.

The reasons for a bad fermentation can then be classified as either having not enough cells or having lots of cells which are poor producers of the desired product.

2) To control the fermentation. If a control strategy is being used to control substrate addition to the fermentation then often the amount of cells present is used to determine how much substrate to add. The ability to measure cell concentration on-line is particularly important for this application.

- 3) To apply *post-facto* analysis and diagnosis. The analysis of fermentations and the development of models often requires the cell concentration. Usually this analysis is conducted after the fermentation.
- 4) To time inoculum addition. It is well known that inoculating a fermentor with rapidly (usually exponentially) growing cells decreases the lag phase and leads to a better fermentation. Timing of inoculum addition is difficult to perform if the growth rate is not known.
- 5) To detect the occurrence of deleterious effects. Measurement of the growth rate of the culture can be used to determine substrate limitation, toxic product inhibition of growth and sudden changes in operating conditions, for example temperature and pH. Growth rate is often used to assess the "well-being" of the cell population.
- 6) To determine the phase of the fermentation. The only conclusive way to detect the onset of the stationary phase is to measure cell concentration. The onset of the stationary phase is important as many products are formed only in the stationary phase. Detection of the stationary phase may influence the decision to harvest the fermentation.
- 7) To characterize new strains, or operating conditions or to formulate media. If cells are a desired product, comparison and selection of strains or medium components often depend on which fermentation produces the most growth.

Many industrial fermentation media contain solid substrate. The problem is that almost all techniques for estimating cell concentration do not work when solid substrate is

present. Carleysmith and Fox, 1984 summarize this frustrating difficulty by stating that "cell mass is notoriously difficult to measure in industrial fermentation media containing solids and oils".

This problem has several implications. The first is the lack of fundamental data. Such data includes cell and solid concentration (once the cell concentration is determined the solid concentration can be determined by subtraction of the cell concentration from dry weight measurements), cell growth rate, solid degradation rate and specific productivity. Without this data, relationships between key parameters cannot be derived, nor can models be developed to describe the fermentation. The end result is that control schemes cannot be implemented. Thus no logical strategies can be devised to improve the fermentation.

One way to overcome this lack of data is to conduct a series of batch fermentations with different amounts of solid initially present in the medium. The medium composition that gives the highest product titer is then chosen for scale up (Hafiz and Qadeer, 1986). This empirical approach to the problem leads to the least harmful initial solid substrate concentration. The important factor in solid substrate fermentations is not the solid substrate concentration but the availability of soluble assimilatable compounds derived from the solid substrate throughout the fermentation. The effects of repression and inhibition by the products of solid degradation are not considered by the batch addition of solid substrate. The batch fermentation is stuck with one solid substrate utilization pattern with little concern for the concentration of degradation products during the fermentation.

The problem addressed in this thesis is a three-tier one. First there is a lack of cell concentration data. Second, since there is no cell concentration data, there is no derived

fundamental data such as growth rate and specific productivity. Relationships between these fundamental data cannot be determined. Thirdly, since there is no fundamental data or relationships available and no models to describe the fermentation, there are no logical approaches to controlling and improving the solid substrate fermentation.

1.3) Problem Statement

- 1) Few techniques, especially on-line techniques, are available to estimate cell concentration in the presence of solid substrate.
- 2) Lack of cell concentration data means relationships between variables that describe the fermentation are not available.
- 3) The lack of characterization of solid substrate fermentations means that there are no logical strategies available to control and improve the fermentation.

1.4) Objectives

- 1) To develop a novel technique to estimate cell concentration in the presence of typical fermentation solid substrates.
- 2) To use the novel technique and other techniques to evaluate product formation.
- 3) Identify the implications that the regulation of product formation has on the operation of the fermentor.

1.5) Model System

The model system for this study was designed to meet the following criteria:

- 1) The organism must be capable of degrading solid substrates. This usually means that the organism must excrete a hydrolyzing enzyme.
- 2) The organism must exhibit constant morphology under varying conditions. This restriction was chosen to eliminate the ill-defined influence of variable cellular morphology (for example, the morphology of filamentous organisms). Variable cellular morphology would make studying light scatter behavior much more difficult. The best cell system would be a unicellular organism.
- 3) A generic system must be studied. This meant the solid substrate must be typical of solid substrates used in industrial fermentations and the fermentation must be representative of a variety of fermentations. This restriction was to make the results of this study applicable to a wide range of fermentation processes.
- 4) The cell/solid substrate combination must exhibit invariant regions in the light scatter spectrum. This criterion was used to select a cell/solid substrate combination that would enable cell concentration to be determined using the light scatter methodology. This criterion was applied after initial screening of a number of such systems was completed.

The model system chosen was protease production by *Bacillus subtilis* var *sakainensis* ATCC 21394 grown on fishmeal as the sole nitrogen source.

This model system meets all the above criteria. *Bacillus subtilis* produces large amounts of protease which are capable of hydrolyzing protein based meals. *Bacillus subtilis* is a unicellular organism. The fermentations involved in this study never progressed to the point of spore formation so this morphological variable was not a factor. Fishmeal is a typical protein-based solid substrate and is commonly used in industrial fermentation media. In addition *Bacillus subtilis* var *sakainensis* ATCC 21394 has been used in industry, is well characterized, and has been proven to grow on fishmeal (Murao, 1971). Proteases are industrially important and are commonly used in detergent preparations.

This fermentation is representative of a range of fermentations that use organisms exhibiting unicellular morphology and utilize solid substrates in the fermentation medium. For example, protease (Ward, 1985), amylase (Fogarty and Kelly, 1980), tyrothricin, gramicidin, polymyxin, bacitracin, licheniformin (Egorov, 1985) and steroid transformations (Imada and Takahashi, 1980) could utilize the growth of a unicellular organism on solid substrate.

In preliminary experiments the *Bacillus subtilis*/fishmeal system exhibited a well-defined invariant region in the light scatter spectrum. This was the main reason for selecting fishmeal. It should be noted however, that other systems also exhibited clearly defined invariant regions, for example *Bacillus subtilis*/soybean meal. The *Bacillus subtilis*/fishmeal system was chosen simply because it exhibited the widest and most well defined invariant regions.

In retrospect this system had one disadvantage. This is related to cellular morphology. At high growth rates in the beginning of the fermentation the cells formed

chains up to 10 cells per chain. This in effect changed the morphology from that of unicellular organism to that of a pseudo-filamentous organism at high growth rates. Fortunately, this phenomenon lasted only a brief time (a few hours after growth began) and did not occur at industrially significant cell concentrations (usually it occurred below 2-3 gDCW/l).

2) LITERATURE SURVEY

2.1) *Bacillus subtilis*

Bacillus subtilis is an industrially important organism and is used in the production of amylases, proteases, and peptide antibiotics (Bulla and Hoch, 1985). It is also an important organism in genetic research and for the production of recombinant products. For example, tissue plasminogen activator has recently been cloned in *Bacillus subtilis* (Wang et al., 1989). *Bacillus subtilis* is popular because it can be tailored to excrete cloned products into the fermentation broth and because it is a safe organism with no known pathogenic interactions with man or animals (Old and Primrose, 1985).

Bacillus subtilis is a motile, endospore forming, gram positive, aerobic bacterium (Sneath, 1986; Gordon, 1977; Collins and Lyne, 1976; Leifson, 1960). It is commonly found in soil (Norris et al., 1981). The life cycle of *bacillus subtilis* consists of two phases: vegetative growth and sporulation. Sporulation typically occurs under adverse conditions and there is only one spore per sporangial cell. The spores are heat resistant and can be seen clearly using a phase contrast microscope (Sneath, 1986; Bulla and Hoch, 1985).

2.1.1) *Bacillus subtilis* var *sakainensis* ATCC 21394

The strain *Bacillus subtilis* var *sakainensis* ATCC 21394 has been described in the patent literature. The rods of the vegetative cells are 0.8-1.0 by 2.5-3.5 microns. In some cases rods 0.8-1.0 by 5.0-6.0 microns or filaments have been observed. The spores are circular to cylindrical 0.7-1.0 by 1.0-1.3 microns. It has been shown to grow on a wide range of solid substrates including fishmeal (Murao, 1971).

2.2) Solid Substrates Used in Fermentation Medium

Based on the composition of microorganisms, fermentation medium must contain sources of carbon and nitrogen along with a wide range of minerals and in some cases vitamins and other growth factors. The first systematic study of fermentation media components was carried out between 1868-1880 by Naegeli who studied which forms of carbon, hydrogen and nitrogen could be most easily utilized by bacteria (Difco Laboratories, 1984). Initially solid components were first hydrolyzed before being added to the fermentation medium. The first protein hydrolyzate commercially manufactured as a fermentation medium component was the manufacture of peptone in 1914 (Difco Laboratories, 1984). With the advent of large scale submerged culture during penicillin production efforts during and shortly after the second World War, solid substrates began to be added to the fermentation medium directly.

Ratledge 1977, lists the main criteria for medium component selection as availability, fermentability and cost factors. Industrial fermentation media often utilize solid substrates because they are widely available, provide a wide range of complex nutrients and because

they are less expensive than soluble media. Fermentations which produce high value products or have high separation and downstream processing costs rarely use solid substrates. This is because of the added difficulty of separating the product from the solid and from the products of solid degradation. The most important classes of industrial fermentations that use solid components are antibiotic fermentations and enzyme fermentations.

The main types of solid used in fermentation medium are either starch or protein based meals. Corn, wheat, rye, sorghum, barley, rice and oats are common sources of solid starch. Protein based meals include cottonseed meal, peanut meal, soybean meal, linseed meal, fish meal, meat and bone meal, blood meal, corn germ meal and corn gluten meal (Zabriskie et al., 1982). Most of the meals are byproducts from other industries. For example, fish meal is dried ground fish or fish waste and the meals of vegetable origin are the residues of oil extraction processes (Cejka, 1985).

Solid substrates can have both positive and negative effects on the fermentation performance. For example, fishmeal has been found to be essential for the production of high levels of some antibiotics (Miller and Churchill, 1986). Gossypol, which is released from cottonseed meal by the action of proteases has been shown to inhibit the growth of *Bacillus subtilis* (Khandeparkar, 1981) and also to stimulate the production of amphotericin B by *Streptomyces nodosus* (Linke et al., 1974).

The model solid substrate chosen for this study was fishmeal. Fishmeal was chosen because of its desirable light scatter properties and also because it is typical of solid substrate used in industry.

2.2.1) Fishmeal

The main use of fishmeal is as an animal feed (Beermann and Hogue, 1986; Agudu, 1971). Fishmeal is made by boiling the fish at 95-100°C. The material is then pressed in a screwpress where the insoluble phase (the press cake) is separated from the liquid phase which contains oil and nitrogenous liquid. The oil is separated by centrifugation and the nitrogenous liquid (called stickwater) is concentrated by evaporation. The press cake and the stickwater are then mixed and dried to produce the final fishmeal product (Lunde, 1968). Ironically fishmeal has been used in the commercial production of proteases and proteases have been used to reduce viscosity and hence save costs in the concentration of stickwater (Schaffeld et al., 1989; Jacobsen, 1985; Jacobsen and Lykke-Rasmussen, 1984).

Historically the fermentation of fish has been used in the production of various Southeast Asian food products (Adler-Nissen, 1986). The use of fish protein in fermentation medium began to get serious consideration in the 1960's. This began with the production of fish peptones for use in fermentation medium (Alder-Nissen, 1986; Sen et al, 1962; Sripathy et al., 1962). In the late 1960's and early 1970's there was much discussion of the world food protein shortage (Adler-Nissen, 1986). As a result many new protein sources were actively sort out and studied, particularly the use of industry by-products. Fishmeal use thus gained popularity until its two main disadvantages became apparent.

Fishmeal hydrolyzates had a bitter and fishy off-flavor taste which precluded their use in any food or food-related products (Adler-Nissen, 1986; Hevia and Olcott, 1977). Secondly there was a major odor problem, not just in the manufacture of fishmeal and in the fermentation plant but in the odor being transferred into the final fermentation product.

This led to fishmeal being abandoned as a fermentation substrate in the production of detergent proteases (Aunstrup, 1980). Washed clothes smelling of rotten fish were not highly admired by consumers.

The composition of fishmeal depends on the fish used for its preparation. The detailed composition of fishmeal made from a variety of fish types can be found in the literature (Miller and Churchill, 1986; Zabriskie et al., 1982; Rao and Kamasastri, 1971; Sen and Keshava, 1971; Arnesen, 1969; Lunde, 1968). As a summary of the above references, fishmeal contains approximately 91-96% dry matter, 40-70% protein, 3-8% fat, 1% fiber, 20-30% ash. Depending on the source and final use contaminants such as sand may be present. Fishmeal contains the elements (though not usually in elemental form) calcium, magnesium, phosphorus, potassium and sulfur. Numerous trace elements are also present. Fishmeal contains the vitamins choline, niacin, pantothenic acid and riboflavin.

2.3) Protease Production

2.3.1) Proteases: The Basics

Proteases are enzymes that catalyze the hydrolysis of proteins (Coombs, 1986). Proteases are classified into three groups; acid proteases which have a maximum activity between pH 2-5, neutral proteases which have a maximum activity between pH 7-8 and alkaline proteases which have a maximum activity between pH 10-11 (Keay, 1972). Proteases are prepared from animal, plant and microbial sources.

The proteases used in detergents (one of the biggest uses of proteases) are most commonly produced by *Bacillus* species (*Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus*

licheniformis and the alkalophilic *Bacillus* species). *Bacillus* species commonly produce a neutral protease which requires a metal ion for activation (metallo-protease) and an alkaline protease which contains serine in the active site (serine alkaline protease) (Aunstrup, 1974). The best known alkaline proteases are the Subtilisins. Subtilisin Carlsberg produced by *Bacillus licheniformis* is the most important detergent protease. Subtilisin BPN (its name is derived from the commercial preparation Bacterial Protease Nagarase) is also used in enzyme detergents. Subtilisin Novo is identical to Subtilisin BPN (Ward, 1985). Subtilisin Carlsberg and Subtilisin BPN differ by only 58 amino acids (Kalisz, 1988). The molecular weight of bacterial proteases is approximately 30,000 daltons (Reed and Underkofler, 1966).

2.3.2) The Development of Protease Production

Proteolytic milk clotting enzymes have been used since about 5000 BC to transform milk into cheese (Ward, 1985). Industrial production of purified, standardized proteases started in 1874 when Chr. Hansen manufactured a rennet preparation in Copenhagen (Aunstrup, 1974). Microbial protease production began on an industrial scale in 1890 when Takamine came from Japan to the U.S.A. and started the production of the enzyme Takadiastase (Aunstrup, 1980). This enzyme was made by the semi-solid fermentation of wheat bran by *Aspergillus oryzae* (Takamine, 1894). Takadiastase was an α -amylase preparation but contained considerable amounts of protease and was used mainly as a digestive aid (Aunstrup, 1980).

The first commercial use of *Bacillus subtilis* for protease production was in 1913 (Boidin and Effront, 1917). This enzyme was primarily used for the desizing of textiles

(Aunstrup, 1980). The use of enzyme detergents also began in 1913 when the German chemist Otto Röhn obtained a patent for a presoaking product called Brunus®, which contained enzymes from animal pancreas glands (Barfoed, 1983).

The 1950s saw the manufacture of microbial proteases switch from solid state fermentation to submerged culture (Aunstrup, 1980). It was not until 1959, however, that the Swiss company Gebrüder Schnyder sold the first detergent to contain a bacterial protease which was made using *Bacillus subtilis* (Barfoed, 1983; Aunstrup, 1980). The first alkaline protease containing detergent, Acalase® was developed in 1960 by Novo Industri in Denmark (Barfoed, 1983, Ward, 1983). Bacterial enzyme detergents began test marketing in the U.S.A. in 1966 (Ward, 1983).

There were two significant developments in the fermentation of alkaline proteases. The first was in the early 1970's with the introduction of a fed-batch strategy for the addition of carbohydrate (Kalabokias, 1971). Amazingly this technique had been tried much earlier but was overlooked by the industry (Güntelberg, 1954). The aim of the fed-batch strategy was to keep the concentration of reducing sugar low (4-10 g/l) throughout the fermentation to avoid repression of protease formation but at the same time provide enough carbohydrate for growth and other metabolic needs.

The second major development was also in the early 1970's with the introduction of non-sporulating mutants (Churchill and Buss, 1973). Non-sporulating mutants avoided the problem of spores contaminating the final detergent product and ending up on clothes. Also non-sporulating mutants led to improved protease yields (Aunstrup and Ottrup, 1973). This was probably due to the fact that they permitted a longer production phase (Aunstrup,

1980). Strain development continued to play an important role with the selection of strains unable to produce undesirable antibiotics, polymers or other products (Aunstrup, 1980).

The use of proteases in detergents increased in the 1960s until in 1969 fifty percent of the detergents manufactured in Europe and the U.S.A. contained proteases (Aunstrup, 1980). The use of enzymes in detergents suffered a setback however, when between 1967 and 1970 incidents of pulmonary disease caused by workers handling enzymes in detergent factories were reported (Aunstrup, 1980). This caused public alarm and caused a recession in the enzyme detergent market after 1969 (Ward, 1983). In 1971 the Ad Hoc Committee on Enzyme Detergents, formed by the Medical Sciences Division of the United States National Academy of Sciences reported on "Enzyme Containing Laundering Compounds and Consumer Health". The report stated that there was no evidence that enzyme detergents were any more dangerous than non-enzyme-containing detergents. The U.S. Food and Drug Administration accepted the report and enzyme detergent sales climbed (Ward, 1983). Shortly after this the problem of enzyme dusts in factories was alleviated by the use of encapsulation technology to produce dust-free granulates (Aunstrup, 1980).

The next development in microbial protease production was the utilization of alkalophilic *Bacillus* species. These species produce alkaline proteases which are stable at high pH, some having maximum activity as high as pH 12 (Aunstrup, 1980). These proteases were better suited to the washing process but they have been used only to a limited extent in detergents because of the strict regulations, cost, and lengthy time required to test and market new enzyme products (Aunstrup, 1980).

Another development was the introduction of microbial proteases as a replacement

for calf rennet in the manufacture of cheese. K. Arima and coworkers in 1960 screened a large number of organisms and found that a protease from *Mucor pusillus* had suitable characteristics for cheese manufacture. In 1963 and 1965 respectively it was discovered that proteases from *Endothia parasitica* and *Mucor miehei* had characteristics that were suitable for cheese making (Aunstrup, 1980).

Today proteases are one of the most important industrial enzymes (Layman, 1986) and they account for close to 60% of total enzyme sales worldwide (Kalisz, 1988). The three major manufacturers of proteases are Novo Nordisk A/S (formerly Novo Industri A/S) in Denmark, Gist-brocades in the Netherlands and Miles Laboratories in the United States. Together these companies control 70% of the industrial enzyme market (Kalisz, 1988).

The main applications of proteases are in detergents, protein hydrolysis and cleaning, soy sauce production, cheese manufacture, brewing, baking, leather industry and in medicine (Ward, 1985).

2.3.3) Protease Production Technology

Most *Bacillus* species, including *Bacillus subtilis*, produce alkaline proteases towards the end of logarithmic growth (Kalisz, 1988; Keay et al., 1972). This production continues during the stationary phase until the culture sporulates. Despite extensive studies (Priest, 1977) the exact control of protease production and its relation to sporulation is still unclear. It is now generally thought that while sporulation accompanies protease formation and excretion, sporulation processes are not essential for protease formation. Rather the

formation of proteases requires the same triggering mechanism responsible for the initiation of sporulation (Ward, 1985). Non-sporulating mutants based on interrupting the sporulating process at various steps have been used to increase protease production and to decrease the chance of protease production being stopped by sporulation (Churchill and Buss, 1973).

Enzyme production by one strain of an organism varies widely with the medium used (Keay et al., 1972). Therefore medium formulation plays an important role in protease production. A typical fermentation medium may contain 100-150 g/l of dry ingredients (Aunstrup, 1980). Various carbon sources have been used in industrial medium including starch hydrolyzates, whey, lard oil (Feldman, 1971), glucose-maltose syrup, molasses, glucose, dextrine, sucrose, glycerin (Murao, 1971), corn starch hydrolyzate, corn steep liquor (Smythe et al., 1950), corn syrup (Noe and Beckhorn, 1968), starch (Nijenhuis, 1977), and cottonseed flour (Vicararo, 1973). Many different nitrogen sources have been used in industrial medium including fishmeal, cottonseed meal (Murao, 1971), soybean meal (Aunstrup and Outtrup, 1970), peanut meal, urea (Aunstrup et al., 1973), wheatbran (Fukumoto et al., 1974), and casein (Churchill and Buss, 1973). The above lists of medium components are by no means exhaustive.

Repression plays a major role in protease formation. The formation of protease is repressed by glucose (carbon catabolite repression) and amino acids (Patterson-Curtis and Johnson, 1989; Klimov et al., 1988; Liebs et al., 1988; Ogrydziak et al., 1977; Heineken and O'Connor, 1972; May and Elliot, 1968; Levisohn and Aronson, 1967; Chaloupka and Kreckova, 1966a, 1966b; Neumark and Citri, 1962). Heineken and O'Connor, 1972 reported that both alkaline and neutral proteases were repressed similarly under the same conditions.

There have been three strategies applied to overcome repression. The first strategy is the use of slowly metabolized carbon or nitrogen sources (Gusek et al., 1988). This is one reason why solid substrates are used as carbon and nitrogen sources, because they are slowly broken down.

The second strategy is by the fed-batch addition of the carbon source, usually glucose, and/or the nitrogen source, usually ammonia (Motai et al., 1989; Kole et al., 1988a,b; Kalabokias, 1971; Güntelberg, 1954). Güntelberg, 1954 kept the glucose concentration between 25-55 g/l. Kalabokias, 1971 aimed to keep the reducing sugar concentration between 4-10 g/l. Kole et al., 1988a,b controlled glucose at 0.15 g/l and ammonium at 5 mmol/l. Motai et al., 1989 kept the total extracellular nitrogen at or below 0.05%.

Güntelberg, 1954 used a predetermined feed profile with no on-line determination of the correct glucose feed rate. Güntelberg, 1954 simply chose to add glucose initially at a steadily increasing rate (2.5 times increase in the rate of addition per hour) followed by addition at a constant rate. The author was primarily interested in making sure that the glucose did not run out as opposed to a consideration of repression effects. Such a strategy with no on-line feedback was the cause of the poor glucose control obtained by Güntelberg, 1954.

The feeding strategy of Kalabokias, 1971 is deliberately not revealed as is common in the patent literature. Kole et al., 1988a,b was able to achieve a very low glucose concentration based on glucose addition whenever the dissolved oxygen went above 20% saturation due to glucose limitation. Control of glucose in this manner led to very low glucose concentrations but this may not be the best manner to control glucose because the

cell population is continually oscillating between glucose being limiting (the DO rising) and glucose being in excess (the DO falling). Although not specifically mentioned with respect to protease production, off-gas analysis, primarily based on oxygen and carbon dioxide composition, are now routinely used to control carbon source supply (Suzuki et al., 1986).

Kole et al., 1988a,b used an ammonium gas electrode to control the addition of ammonium sulfate to the fermentation broth. Güntelberg 1954, fed casein digest in the same solution as the glucose feed. Motai et al., 1989 fed a protein containing liquid to control the total extracellular nitrogen level.

No references could be found in the literature detailing a fed-batch strategy for solid substrate addition in submerged culture. The fed-batch control strategies employed in industry are restricted, in-house information. Traditionally, however, solid substrate has been added in a batch fashion. Simple experiments are performed with increasing batch concentrations of solid substrate. The concentration that leads to the highest product titer is chosen for larger fermentations (Hafiz and Qadeer, 1986). Such a strategy gives no consideration to the effects of repression and the repressor concentrations during the fermentation. This strategy leads to the least harmful initial solid substrate concentration.

The third strategy to overcome repression has been the use of continuous culture. Continuous culture can be used to keep the concentration of glucose and amino acids at very low levels and also provides a means of controlling the growth rate. Several authors (Allison and MacFarlane, 1989; Fukushima et al., 1989; Pazlarova and Tsaplina, 1988; Heineken and O'Connor, 1972) have compared continuous culture with batch culture on a laboratory scale for the production of protease. Continuous culture for protease production

has yet to be used on an industrial scale. The reason for this is that the fed-batch process is more economic because of the inefficient utilization of medium in the continuous process (Aunstrup, 1980). Also the production of proteases in continuous culture has been shown to be prone to contamination.

Keay et al., 1972 has stated that, potentially, protease fermentations could be improved by changing the medium composition or conditions at some point midway through the fermentation. The conditions in the first phase of the fermentation would favor cell growth while the conditions in the second phase of the fermentation would favor enzyme excretion. Such an operating strategy for protease production has yet to be demonstrated in the literature.

The fed batch addition of solid substrate to protease fermentations to avoid amino acid repression and viscosity effects has not been documented in the literature but industry is known to conduct fed batch addition of solid substrate (Hjortkjaer, 1990). One of the few studies on the fed batch addition of non-soluble substrate during fermentation was the feeding of soybean oil during the production of emulsan (Shabtai and Wang, 1990). Soybean oil was fed in coordination with base addition for pH control to maintain a fixed carbon to nitrogen ratio in the feed.

Bacillus species are known to produce a variety of non-enzyme products during aerobic cultivation including acetate, acetoin, butanediol, ethanol and lactate (Delgado et al., 1989; Mas et al., 1988; Moes et al., 1985; Mahmoud et al., 1975; Lopez and Fortnagel, 1972; Mahmoud, 1971). The product distribution from *Bacillus subtilis* is sensitive to dissolved oxygen concentration and *Bacillus subtilis* culture has been used to characterize

oxygen transport in bioreactors (Moes et al., 1985).

These end-products have been shown to inhibit the growth of *Bacillus*. Mas, 1988 showed that acetate, acetoin, 2,3-butanediol, ethanol, glucose and lactate inhibit the growth of *Bacillus polymyxa*. Table 1 shows the concentration of each toxic product and glucose required to completely stop growth. Acetate and acetoin are the most toxic to the cell and 2,3-butanediol the least toxic product. Acetic acid has been shown to be very toxic in numerous other bacterial systems and the undissociated form of the acid identified as the most inhibitory species (Reis et al., 1990; Tang et al., 1989; Wang and Wang, 1984; Leung, 1982).

Table 1: Relative strength of toxic products and substrate on the inhibition of the growth of *Bacillus polymyxa* (data from Mas et al., 1988).

Product or Substrate	Lowest Concentration Required to Completely Stop Growth (g/l)
Acetate	7
Acetoin	20
Ethanol	45
Lactate	50
2,3-butanediol	90
Glucose	190

2.3.4) Notes on Reading the Protease Literature

The literature contains a number of good review articles on protease production to which the reader is referred if additional information is required (Kalisz, 1988; Ward, 1985; Ward, 1983; Barfoed, 1983; Debabov, 1982; Starace, 1981; Aunstrup, 1980; Starace and Barfoed, 1980; Aunstrup, 1974; Meltzer, 1973; Keay et al., 1972; Keay, 1971; Reed and Underkofler, 1966; Hagihara, 1960). When reviewing the literature keep in mind that there was a classification confusion regarding *Bacillus*. Prior to 1970 (approximately) many protease producing *Bacillus* strains were poorly classified as *Bacillus subtilis*. This was because the old definition of *Bacillus subtilis* was not very detailed. Subsequently small differences between these organisms became apparent (Welker and Campbell, 1967) which resulted in a new classification definition which split the old *Bacillus subtilis* class into *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus pumilis* (Aunstrup, 1974). Also note that most of the papers and patents describing the industrial production of proteases are published prior to 1975. After 1975 very little information on industrial processes is available, most of it remaining as in-house data. Hence, data on production figures and techniques are probably out of date.

2.4) Measuring Cell Concentration in the Presence of Solid Particles

The cell concentration measured in most fermentations is the total cell mass including viable (capable of reproduction) and non-viable cells. Very few techniques distinguish between viable and non-viable cells.

There are many good reviews of the numerous techniques that have been devised to

measure cell concentration in medium with no solid particles present (Phillips, 1990; Omstead et al., 1990; Baserga, 1989; Agar, 1985; Harris and Kell, 1985; Karube, 1985; Onken et al., 1985; Carleymith and Fox, 1984; Joglekar et al., 1983; Cooney, 1981; Wang et al., 1979; Pirt, 1975). Conversely there are only a small number of papers dealing with the measurement of cell concentration in the presence of solid particles. This discussion will be limited to those techniques which have been shown to estimate cell concentration in the presence of other solid particles.

The approaches to measuring cell concentration in the presence of other solids can be classified as follows:

- 1) Direct microscopic observation.
- 2) Colony counting on a plate.
- 3) Prior separation of the cells and solids.
- 4) Preferential dissolution of the solid.
- 5) Measurement of a selective component that is not present in the solid.
- 6) Methods that lyse the cells but do not affect the solid.
- 7) Substrate utilization or product formation.
- 8) Measurement of a physical property that is different in the cell and the solid.
- 9) Deconvolution of light based data.

1) Direct microscopic observation

In this technique the cells and solid are viewed under a microscope and various techniques are used to aid in counting the cells. An example in a bacterial system is the cell

counting of *Zymomonas mobilis* in the presence of soy flour by the use of a Petroff-Hausser bacterium counter (Ju et al., 1983). Examples involving yeast include the cell counting of *Saccharomyces cerevisiae* in the presence of whole soy flour using a Petroff-Hausser bacterium counter (Damiano and Wang, 1985) and the counting of *Saccharomyces carlsbergensis* in the presence of unrefined soybean flakes using a Levy hemocytometer (Kleyn and Vacano, 1966).

The ease of differentiating the cells from the solid can be enhanced by using a staining technique. The stain is usually a fluorescent dye or a fluorescently labelled antibody. For example, the use of epifluorescence microscopy to detect *Thiobacillus ferrooxidans* in the presence of ore particles by using an acridine orange stain (Yeh et al., 1987) and the use of fluorescent antibody staining to detect *Thiobacillus ferrooxidans* attached to the surface of coal refuse (Apel et al., 1976).

Cell distribution on solid particles can also be determined using a scanning electron microscope. For example, the colonization of *Thiobacillus ferrooxidans* on support matrix materials was studied using a scanning electron microscope (Grishin and Tuovinen, 1989). Determination of cell concentration on a routine basis using a scanning electron microscope is not practical.

The disadvantage of direct microscopic observation is that in some cases the cells attach to the solid surface and this makes counting difficult. Also the technique is very labor intensive and operator fatigue can decrease accuracy. Cell enumeration techniques are usually a "last resort" approach to the problem.

2) Colony counting on a plate

Colony counting on a plate takes advantage of the fact that the cells will grow and produce colonies whereas the solid particles will not. A dilute suspension is used to inoculate a plate and the colonies are counted after incubation. For example the estimation of *Thiobacillus ferrooxidans* in the presence of ore particles (Espejo and Ruiz, 1987). Colony counting suffers from the disadvantages of all cell enumeration techniques (listed above).

3) Prior separation of the cells and solids

This technique first separates the cells and the solid, then uses any of the standard cell concentration estimation techniques to quantify the amount of cells present. This method is not applicable in most cases because it is very difficult to separate the cells and the solid. However, there are three notable exceptions.

The first occurs when there is a large difference in density between the cell and the solid. In this case the cells and solid can be separated by centrifugation then the cells recovered. A case where the solid is much more dense than the cells is the separation of solid ore from *Thiobacillus ferrooxidans* (Espejo and Ruiz, 1987). A technique used when the density of the cell and the solid are similar, is to use centrifugation followed by freeze drying of the solid pellet. After freeze drying the cells layer can be cut from the solid layer more easily and then a dry weight analysis performed on the cells (Suzuki, 1988). Obtaining a distinct cell/solid interface and completely separating the cells and the solid are problems when using this technique.

The second exception is by the use of filtration to separate the cells and solids. For

example, the filtration of blended food samples containing *Salmonellae* using a 35 micron nylon cloth filter (Tsen et al., 1989). The disadvantage of this method is that all solid particles smaller than the filter cut off size will be counted as cells and that some of the cells may adhere to the filter.

The third exception occurs when the solid is very large or contiguous and can be easily removed from the medium. Such a case occurs in anchorage dependent mammalian cell culture. The cells are detached from their solid support matrix by trypsinization (Baserga, 1989). After the cells are in the liquid medium the solid support is removed and the cells counted using a variety of techniques.

4) Preferential dissolution of the solid

If the solid particles can be preferentially dissolved then the remaining cell concentration can be determined using standard techniques. For example, the dissolution of ferric iron precipitates with EDTA enables the concentration of *Thiobacillus ferrooxidans* to be determined with a coulter counter (Schuler and Tsuchiya, 1975). In the growth of cells in a semi-solid gel, the liquefaction of the gel at high temperatures allows the cell mass concentration to be determined using traditional techniques, (Marin-Iniesta, 1989; Wei et al., 1983). Solid calcium carbonate particles can be removed from microbial cultures by washing with mineral acids (Agar, 1985). Solid hydrocarbons can be removed from cell samples by extraction with solvents (Amin et al., 1973; Yamada and Yogo, 1970). Sodium hydroxide and heat are used to dissolve cellulosic residues in cell samples prior to cell mass determination (Huang et al., 1971). Since in most fermentations the properties of the cell

and solid are similar this technique is of limited application.

5) Measurement of a selective component that is not present in the solid

This component must be present in a fixed quantity per unit cell mass and not be present in the solid. This again is more unusual than the norm. Protein content is often correlated with cell concentration when cells are grown in the presence of non-proteinaceous solids. For example, the growth of cells on cellulose can be monitored using a protein assay (Greene and Gordon, 1989; Moreira et al., 1978).

Chitin (poly-N-acetylglucosamine) present in fungal cell walls can be used to calculate cell concentration. For example, the growth of *Aspergillus oryzae* on koji is followed by measuring glucosamine (Ito et al., 1989; Aidoo et al., 1981). Lipid bound phosphates have been used to determine cell concentration in sediments (Findlay et al., 1989).

Mixed culture provides the unique challenge of identifying two populations that are very similar in most properties. In this situation a compound in one cell population must not be present in the other. For example, 8-anilino-1-naphthalenesulfonic acid can be used to distinguish between gram-negative and gram-positive bacteria (Ramsey et al., 1980). Another example is the differentiation of methogens from non-methogenic organisms using coenzyme F₄₂₀ (Mink and Dugan, 1977). The cell concentrations of *Streptococcus cremoris* and *Leuconostoc lactis* can be determined in mixed culture by measuring two different enzymes, each specific to only one strain (Boquien et al., 1989). Gene probes (Steffan et al., 1989) and fluorescent antibodies (Kurane et al., 1979) have been used to estimate cell concentration of specific organisms in mixed culture.

The problem in these techniques is finding a substance that is present in the cells and not the solid and also finding a substance that remains a constant proportion of the cell mass during all phases of cellular metabolism. In practice, for most systems, this is very difficult.

6) Methods that lyse the cells but that do not affect the solid

The principle of these techniques is that the cells are preferentially lysed but the solid remains intact. The mixture is then centrifuged to remove the solid and the cell debris. The supernatant, which contains the cells interior substance, is then analyzed for any substance which is present in a fixed quantity per cell. Sonication or chemical rupture of the cell followed by the measurement of DNA content or total organic carbon are popular choices for these assays (Solomon et al., 1983; Hashimoto et al., 1982). ATP has been extracted and related to cell concentration in the composting of grape pulp and sheep manure (Thierry and Chicheportiche, 1988). Protein has been solubilized from cells by treatment with sodium hydroxide and related to cell concentration in the fermentation of *Aspergillus niger* on cassava meal (Raimbault and Alazard, 1980). Dehydrogenase activity was used to estimate the growth rate of a heterogeneous microbial population growing in swine manure (Ghaly et al., 1989; Ghosh et al., 1972).

7) Substrate utilization or product formation.

The cell mass concentration can be calculated from a substrate consumption rate or a product formation rate. Substrate consumption or product formation is assumed

proportional to cell accumulation. For example, the ammonium sulfate consumption rate is used to calculate the cell mass concentration during the growth of *Trichoderma reesei* on leached beet cosette (Schaffeld and Illanes, 1982). Alpha-amylase and amyloglucosidase concentrations parallel cell mass concentration in *Aspergillus oryzae* fermentations of koji (Aidoo et al., 1981).

The most commonly used method to monitor cell concentration in fermentations which utilize solid substrate is based on the measurement of the inlet and outlet gas composition, and the gas flowrate. The cell concentration can be estimated from oxygen uptake rate or carbon dioxide evolution rate data. For example, the oxygen uptake rate was used to estimate cell concentration in the fermentation of *Aspergillus oryzae* on steamed rice (Sato et al., 1983). The carbon dioxide evolution rate was used to estimate the growth rate during the fermentation of *Aspergillus niger* on cassava flour (Carrizalez et al., 1981) and *Candida utilis* on ryegrass straw (Han, 1987). Another method involves measuring the carbon dioxide evolution rate of soil after fumigation. The carbon dioxide evolution was due to the decomposition of killed microbial biomass by recolonizing populations (Tateishi et al., 1989).

Heat is another product of microbial metabolism and the amount of heat evolved can be used to estimate cell concentration (Marison and von Stokar, 1986; Bayer and Fuehrer, 1982).

All of the methods in this section suffer from the fact that substrate consumption and product formation often do not correlate to cell concentration in all stages of the fermentation. For example, as a culture enters the stationary phase the cell concentration

becomes constant, however, substrate consumption and product formation can still occur.

8) Measurement of a physical property that is different in the cell and the solid.

Methods in this section measure a physical property which is different (usually under set conditions) in the cells and the solid. Since the cells and solid are usually of similar composition and the fermentation medium often interferes, this property is very difficult to find. However, methods of this type offer the most promise for overcoming the current difficulties in estimating cell concentration in the presence of solid substrate.

One promising method is the measurement of radiofrequency dielectric properties to determine cell concentration. For example, the determination of the concentration of *Saccharomyces cerevisiae* in the presence of calcium carbonate (Harris et al., 1987). This method has the ability to be an on-line measurement. This technique has recently been commercialized as the "bugmeter viable cell monitor" (Aber Instruments, 1989). However, the amount of actual fermentation data in the literature, with realistic fermentation substrates, is small.

9) Deconvolution of light based data

Light based data (scatter, absorbance or optical density) are usually not used in the presence of solid particles because the solid particles interact with the light beam. This means that the contribution of the solids must be separated from that of the cells. Thus deconvolution techniques are used to obtain a signal that can be related to cell concentration. The work of this thesis falls into this category.

Wei et al., 1983 was one of the first to apply light based data to estimate the cell concentration in the presence of a solid. Wei et al., 1983 measured the cell concentration of *Saccharomyces cerevisiae* in a 10% solution of solidified gelatin. A small path length, 0.5 cm, was used so that high cell concentrations could be measured. The authors assumption that the gelatin and medium components produced no interfering absorbance was successful. However, this assumption is violated in most other solid-containing media so their technique is of little value.

Hong et al., 1987 extended the technique further and estimated the concentration of *Saccharomyces cerevisiae* in the presence of potato solids. The moisture content of the samples was between 50-95%. After suitable dilution of all the samples to the same water content the authors measured the absorbance of the cell/solid mixtures relative to a blank of cell free solid at the same moisture content. The authors fitted their variable water content data with an interaction model assuming that the measured absorbance was due to a cell component term, a solid component term and an interaction term containing the product of the cell concentration and the solid concentration. The solid component term could be discarded because in their case they measured the absorbance relative to a cell free solid blank.

Hong et al., 1987's technique suffers from several limitations. The technique is only applicable if the solids concentration does not change during the fermentation or if the contribution of the solids to the measured absorbance is much less than that of the cells. The technique also requires dilution and a dry weight analysis so it is not an on-line technique. Since the solid concentration will change significantly in most fermentations and

the solids present in most industrial fermentation media will absorb light strongly this technique is of narrow application. What is surprising about Hong et al., 1987's work is that the authors found a practical system to which their technique applied.

The main disadvantages of most of these techniques are that they are off-line, usually apply under a limited range of conditions and are labor intensive. Of all the techniques available the carbon dioxide evolution rate technique is the easiest to operate on-line and is the industry standard.

2.5) The Application of Light Scatter Techniques to Monitor Fermentation

The application of light scatter and absorbance techniques to cellular systems began early in the 1900s. Bacterial suspensions were compared to standard "opacity tubes" containing barium sulfate (Brown, 1914; Brown and Kirwan, 1914; Brown, 1919). The comparison was made by holding the unknown suspension and the standard side by side against a white card ruled with a black line (raising the tubes slightly from the surface of a clearly printed book was often used). The tubes were viewed by daylight while standing back to a window (Meynell and Meynell, 1970). Later small glass particles (1.5-3 microns long) suspended in water were used as the standard (Maaløe, 1955). The grey-wedge photometer was developed in the 1960s to make comparison easier. In this machine, light from the source reaches the eye by two paths, one through the unknown sample and the other through the standard. The two halves of the field of view are equalized by interposing a wedged shaped filter in one path (Meynell and Meynell, 1970; Scullard and Meynell,

1966).

Apart from the subjective comparison based on the eye, the application of light scatter techniques did not develop to any significant extent until the 1950s when it was noted that the germination of spores caused a drop in turbidity often so marked that it was visible to the naked eye (Powell, 1963; Powell, 1950). By the late 1950's and the 1960's some of the key relationships of absorbance and light scatter by cells were developed. The light scatter intensity was found to be dependent on the refractive index difference between the particles and the suspending medium (Powell, 1963). The application and limitations of the Beer-Lambert Law had been found (Gerrard et al., 1961). The angular and wavelength dependence of light scatter and the effect of particle size had been established (Powell, 1963). Heating the cells was found to affect the light scatter readings (Allwood and Russell, 1969).

It was in this developmental time period that spectrophotometers and nephelometers, designed to utilize the Beer-Lambert Law, came into widespread use for estimating cell concentration. The Beer-Lambert law (West, 1982) states:

$$\frac{I}{I_o} = \exp(-\epsilon.B.C) \quad (1)$$

where

- I : Incident light intensity
- I_o : Transmitted light intensity
- ε : extinction coefficient
- B : path length

C : concentration

In the spectrophotometer, the decrease in light intensity as it passes through a solution is measured and usually translated into an optical density. One particular spectrophotometer, the Klett-Summerson spectrophotometer was so widely used that the Klett unit became a standard method of reporting cell concentrations (Wang et al., 1979). In the nephelometer the light detector is at an angle to the incoming incident light. Automatic dilution techniques have been used to overcome the limitation that Beer's Law does not apply at high cell concentrations (Ramirez and Park, 1990; Imming et al., 1982).

Work on light scatter began to increase in the late 1960s when the angular dependence of light scatter was used to identify bacteria. The angular dependence of the light scatter (called differential light scatter) was found to vary sufficiently between bacterial species so as to be used for identification (Wyatt, 1969). Differential light scattering measurements were later shown to be dependent on the size, shape and structure of the cells (Berkman and Wyatt, 1970). Differential light scatter thus became a tool to investigate cellular morphology (Wyatt, 1972). One of the main applications of differential light scattering is to estimate cell size (Stull, 1972). Cell structure including cell wall thickness and refractive index can also be determined using differential light scatter (Wyatt and Phillips, 1972). For cells under constant morphological conditions, differential light scatter was used to estimate cell concentration (Wyatt, 1970). The angular dependence of the light scatter measurements has many applications today in flow cytometry (Wittrup et al., 1988; Benson et al., 1984; Loken et al., 1976).

The modeling of light scatter data from cell systems remains largely limited to

specific cases. The angular dependence of the light scatter of cell suspensions has been modeled using two approaches. The first approach uses Mie theory which applies to homogeneous particles. Exact solutions are complex, and have been only evaluated for spheres (Kotlarchyk, 1978) and infinitely long cylinders (Morris and Jennings, 1974). Approximate solutions are available for ellipsoids (Wyatt, 1962; Koch, 1961). However, cells are not strictly homogeneous and in the second approach cells are modeled as a cytoplasm with an optically different envelope (Morris and Jennings, 1974). Wyatt, 1970 models the cell as a sphere consisting of a cytoplasm and a cell envelope both of which follow Rayleigh-Gans-Debye theory. The second approach seems to be more effective in predicting cell size (Brunsting and Mullaney, 1972).

Perhaps the most significant development with respect to the application of light scatter to fermentation processes occurred at the end 1972 when the first fiber optic light guide fluorometer was built (Mayevsky and Chance, 1982). Now for the first time the light could be taken to the sample and special fixed sample holders could be avoided. Fiber optics meant that light scatter now became a potentially on-line technique. The development of on-line cell density probes based on turbidity and using fiber optics quickly followed (Hancher et al., 1974; Lee, 1981).

Further application of light scatter to fermentation processes was again driven by developments in fiber optic technology when in the mid 1980s Junker used fiber optic probes of various configurations to estimate cell concentration on-line during fermentation (Junker et al., 1988; Junker, 1988). The advantage of the front face probe was that high cell concentrations could be obtained on-line. Junker et al., 1988's development surpassed that

of Hancher et al., 1974 (even though Hancher et al., 1974 used a front face configuration) because no flow-through sampler was needed, the front face configuration could now be dipped straight into the fermentation broth. Hancher et al., 1974 and Junker et al., 1988 pointed the way for further developments in the on-line monitoring of cell concentrations. Namely the combination of the front face configuration with fiber optics could mean that light scatter could be used for the on-line estimate of industrially significant cell concentrations.

Recently there has been some development of on-line absorbance measurements due to the application of fiber-optics. Dekovich et al., 1989 reports that *Escherichia coli* can be measured on-line up to cell concentrations of 100 g/l using absorbance. Such developments are due to the linearization of absorbance readings using a fourth order polynomial. A correction factor is then applied to compensate for the effect of agitation. Luli et al., 1989 report on a similar device. Again the approach of linearizing the absorbance reading is taken. In this case, for low probe absorbance readings, an exponential curve is used to linearize the result, and at high cell concentrations a fourth order polynomial is used to linearize the result. The advantage of the Luli et al., 1989 probe is that it is designed as an *in situ* sampling/degassing device so that bubbles and agitation effects are avoided. Both the devices (Luli et al., 1989 and Dekovich et al., 1989) are now on the market. The disadvantage of these systems from an industrial point of view is that they are not suitable for use when solid components are present in the medium as solid components will add to the measured absorbance.

There are very few references to measurement of the light scatter of cells in the

presence of solid particles. The reason for this is that the interference from the solid particles is a major application problem (Clarke et al., 1986). Photon correlation spectroscopy (for an overview of this light scatter technique see Brown, 1985) has been shown to detect contaminants during fermentation and also to detect the germination of bacterial spores (Carr, 1988).

3) MATERIALS AND METHODS

3.1) Equipment

3.1.1) Fermentor

The fermentor used in this study was a 5l New Brunswick Scientific BioFlo II (New Brunswick Scientific Company, 44 Talmadge Road, P.O. Box 4005, Edison, New Jersey 08818-4005). The BioFlo II is a glass fermentor with a top entry motor drive. The fermentor assembly was modified to accommodate a balance (a LB-60 balance from Ohaus Scale Corporation, 29 Hanover Rd, Florham Park, New Jersey 07932) underneath the fermentor. The purpose of the balance was to monitor the liquid volume of the fermentor during the fermentation. A photograph of the fermentor can be seen in figure 1.

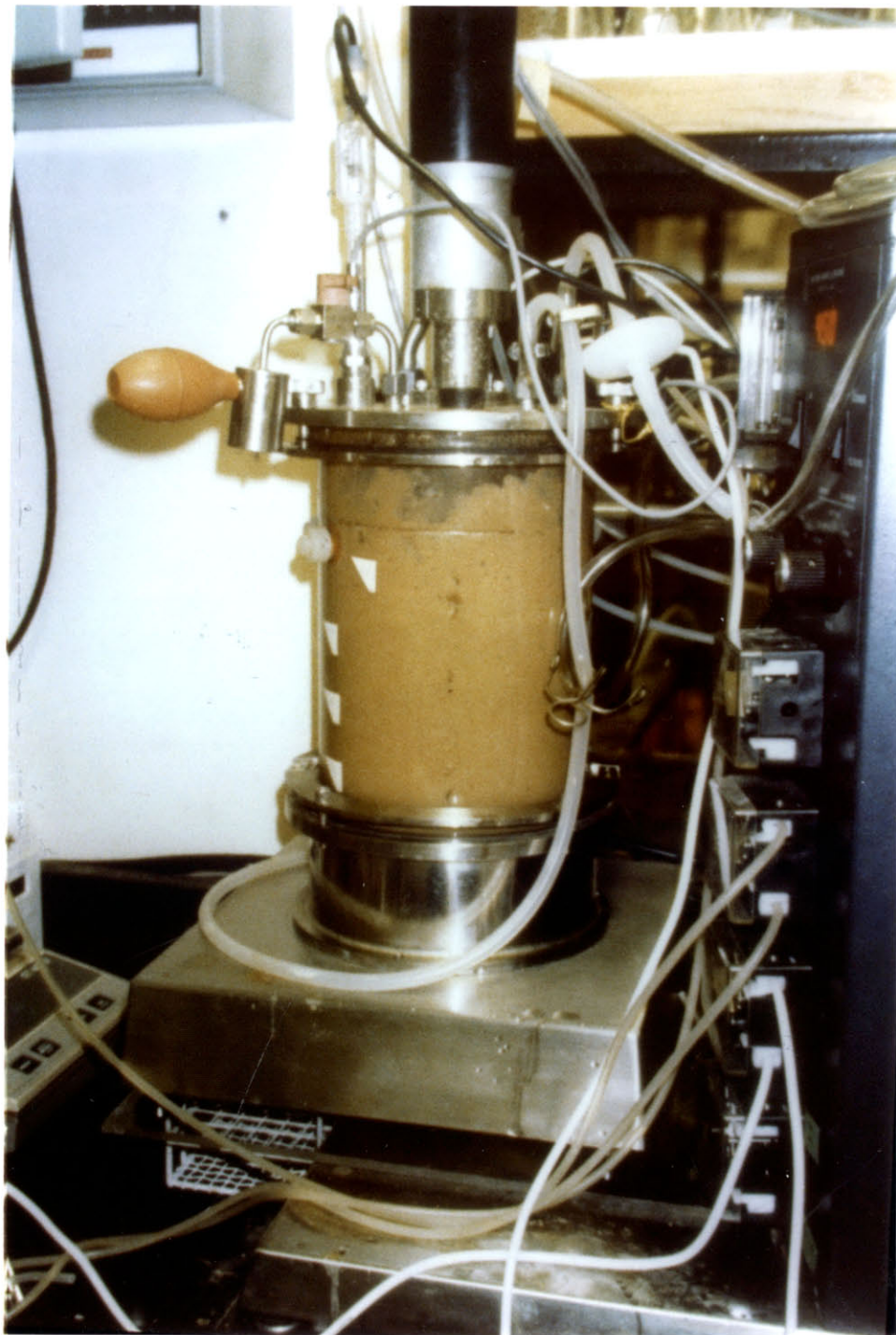


Figure 1: The BioFlo II fermentor and LB-60 scale.

3.1.2) Data Acquisition and Control Systems

Two data acquisition and control systems were used during this study. A FORTRAN program run on a PDP 11/23 computer (Digital Equipment Corporation, 146 Main St., Maynard, MA 01754) was upgraded to a data acquisition software package called Paragon (Intec Controls Corporation, 130 West St., Walpole, MA 02081) run on a PC based system.

The PDP 11/23 computer was programmed in FORTRAN under the RSX11M operating system. The program, which in part relied on the experience and efforts of earlier graduate students in this laboratory, can be seen in appendix A. The signal conditioning used with the PDP 11/23 was a TCS 6432 signal processor (Turnbull Control Systems, 11515 Sunset Hills Road, Reston, VA 22090) which communicated with the PDP 11/23 using RS232 protocol.

Paragon version 3.51 was used on a PC-AT clone (Computer Systems, 4 Fair Oaks Avenue, Newton, MA 02160). The program, which is composed of a series of programming blocks linked graphically on the screen (as well as some subroutines written in C), can be seen in Appendix B. The signal conditioning unit used with Paragon was constructed from individually purchased components, see appendix C. The signal conditioning unit communicated with the PC using RS-422 protocol. The overall Paragon setup can be seen in figure 2 and the Paragon signal condition board can be seen in figure 3.

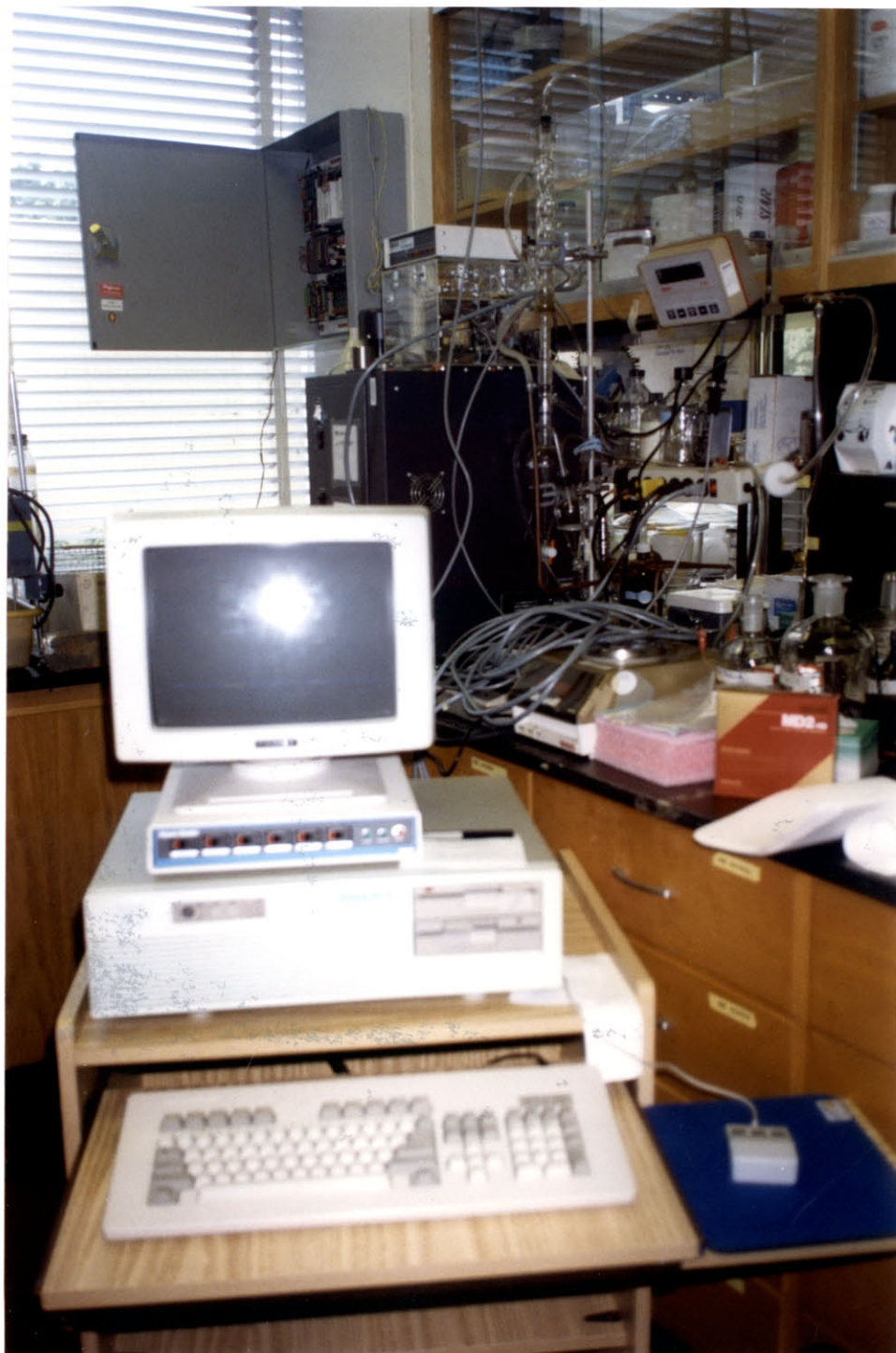


Figure 2: The PC-based control system.

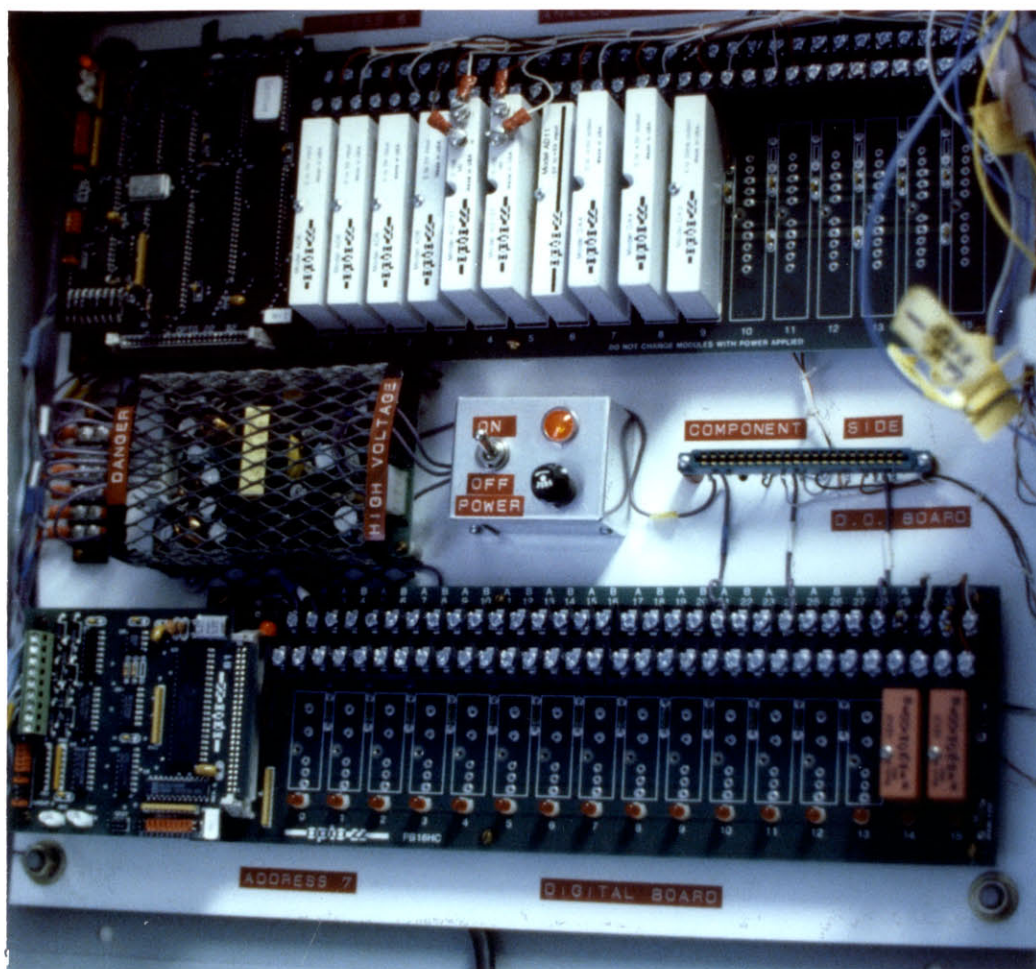


Figure 3: The PC-based control system signal conditioning board.

3.1.3) Fluorometer

All the light scatter measurements were obtained using a fluorometer made from components supplied by the Oriel Corporation (250 Long Beach Blvd., P.O. Box 872, Stratford, Connecticut 06497). A schematic diagram can be seen in figure 4 and photographs of the fluorometer setup in figures 5, 6 and 7. The excitation and the emission monochromator wavelengths were set to be the same so that light scatter was measured. Fluorescence was not measured.

The light source was a 150 Watt Xenon arc lamp. Light from the source first passes through the excitation monochromator. This monochromator permits the passage of light of a narrow range of wavelengths. The slit width of the excitation beam was typically 5 nm and the slit width for the emission beam was typically 20 nm. The wavelength passed by the monochromator could be manipulated to any desired value between 300 and 900 nm. Normally, scans between 330 and 700 nm were taken.

After the light passes through the monochromator it is split into two directions using a beam splitter. One portion of the light goes to the excitation photomultiplier and the other goes on through a fiber optic probe to the sample. Some of the light scattered by the sample then returns via the fiber optic probe and an emission monochromator to the emission photomultiplier.

Both the excitation and emission photomultipliers produce a current whose magnitude is proportional to the intensity of light reaching it. Since the current produced by the photomultipliers is very low, a bias voltage is applied to these currents by the radiometers to amplify them to acceptable readable values. The bias voltage for the

excitation photomultiplier was 300 volts and for the emission photomultiplier, 800 volts. After amplification the current signals were transformed into a digital signal and sent to an Apple IIE computer.

The software used to log the data and to control the fluorometer was Fluorescence Spectroscopy Version 1.1 which was jointly developed by the Oriel Corporation and the Massachusetts Institute of Technology, see Junker, 1988. A copy of the program can be seen in appendix D. The data generated by the fluorometer was converted from APPLE format to IBM format, for further analysis, by Baker Business Services (15 Fenton Avenue, Lynn, MA 01905).

The light scatter readings reported in this study are the ratio of the current produced by the excitation radiometer to the current produced by the emission radiometer and hence are dimensionless. This ratio was used because it meant that variations in source light intensity as a function of wavelength and the decreased intensity of the source as it aged were avoided.

The design of the light scatter probe used in these experiments was crucial. The probe used was a front faced probe (model #77558, Oriel Corporation) which measured light scattered at zero degrees (or backscatter direction) relative to the incoming light. Photographs of the probe can be seen in figures 8 and 9.

Equipment component part numbers and further detailed information about this equipment setup can be found in two previously published descriptions (Junker, 1988 and Junker et al., 1988).

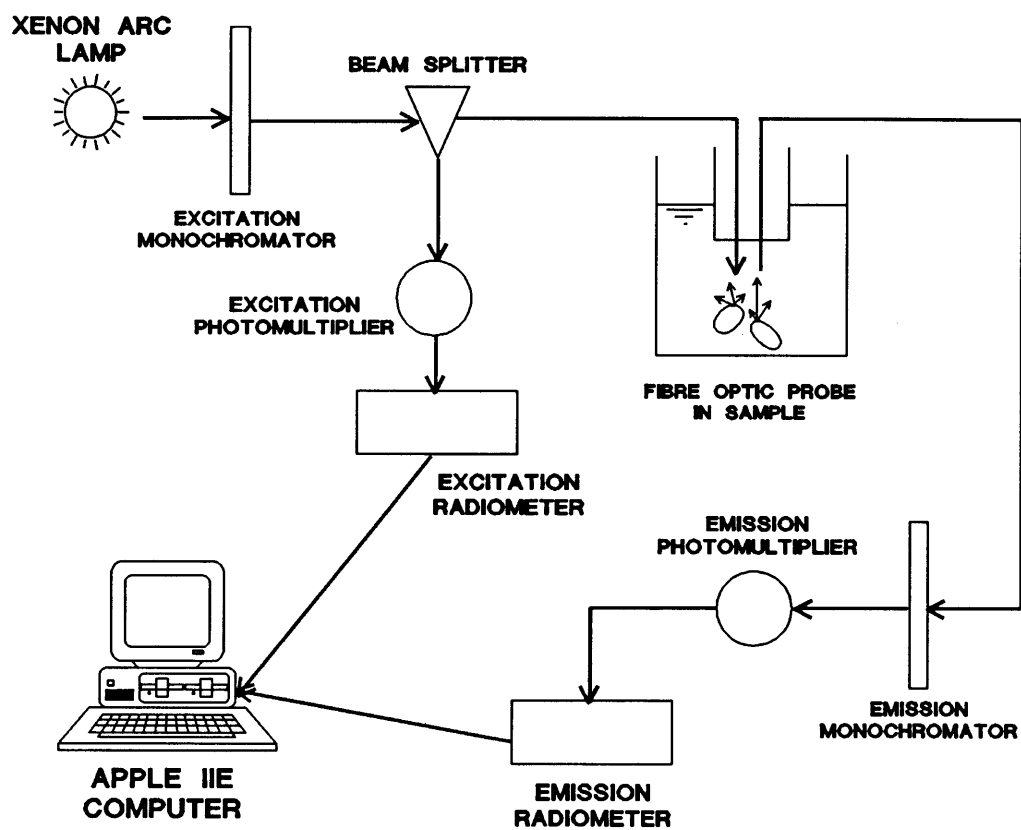


Figure 4: Schematic diagram of the fluorometer and control system.



Figure 5: Overall view of the fluorometer and Apple PC control system.

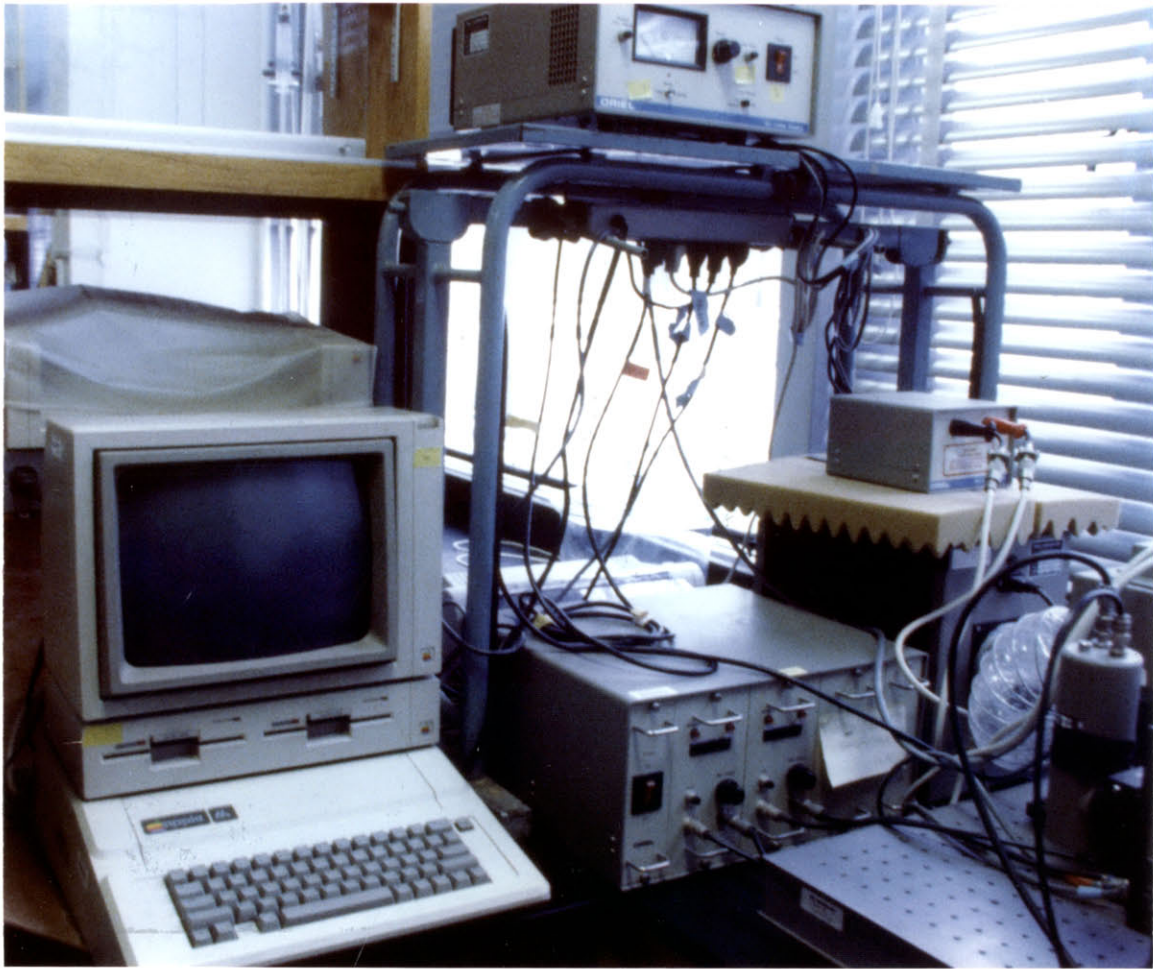


Figure 6: The radiometers, arc lamp supply and Apple PC.

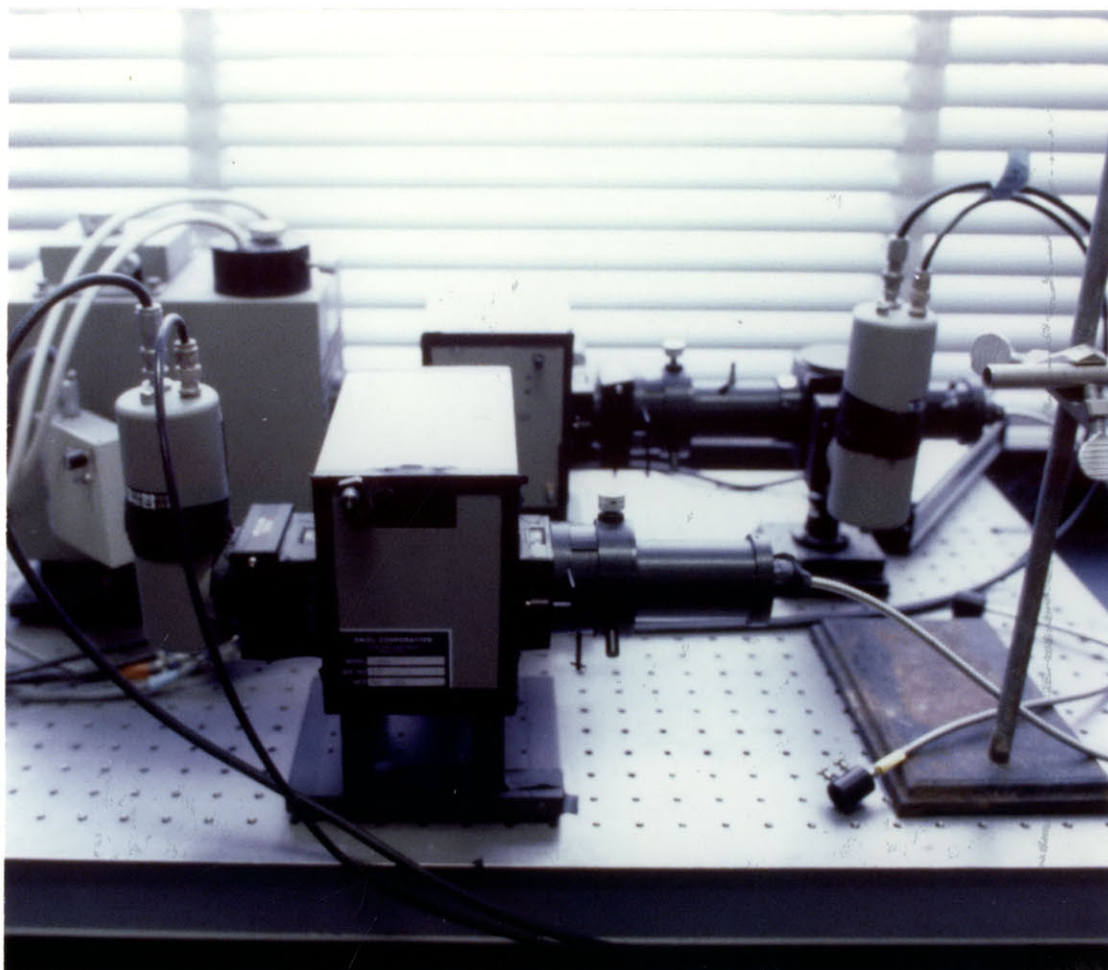


Figure 7: Light train of the fluorometer, including the xenon arc lamp, ozone eater, photomultipliers and fiber optics.



Figure 8: Side view of the front face fiber optic probe showing the excitation and emission fiber optic lines.



Figure 9: End view of the front face fiber optic probe showing the excitation and emission fiber optic lines.

3.2) Assays and Techniques

3.2.1) DNA Assay to Estimate Cell Concentration

This technique distinguishes between cells and solid substrate by sonicating the cells to release DNA while leaving the solid substrate unaffected. The DNA content of the supernatant is then measured fluorometrically and related back to a standard curve which is prepared by growing the cells on soluble substrate.

The fluorescent DNA assay is based on a paper by Brunk et al., 1979. This method has been used extensively and Brunk et al., 1979's initial work has been cited in 175 papers up to 1987. The basis of the assay is the fluorescent dye DAPI (4',6-diamidino-2-phenylindole) which complexes with DNA. The binding of DAPI to DNA is highly specific for adenine-thymine base pairs. The DAPI/DNA complex has approximately 20 times the fluorescent intensity of the dye alone (Brunk et al., 1979). Under the conditions of the assay an increase in fluorescence of a sample over that of a blank is proportional to the DNA content of the sample. A DNA standard is used to calibrate the results.

Both nuclear DNA and mitochondrial DNA are detected by the assay (Williamson and Fennell, 1975). The interference from other cellular molecules is negligible. The fluorescence of the DAPI/RNA complex is very low. On an equal weight basis *Escherichia coli* tRNA has 0.003% of the fluorescence of DNA and yeast RNA has 0.37% the fluorescence of DNA (Brunk et al., 1979). Single stranded DNA is detected but the signal has only one third the intensity of that produced by an equivalent amount of double stranded DNA (Brunk et al., 1979). Proteins and polysaccharides do not give a significant fluorescent enhancement (Brunk et al., 1979). Both ionic and non-ionic detergents do not

interfere with the assay except when the concentration of ionic detergents exceeds 10 g/l. Hence, high concentrations of ionic detergents should be avoided in cell lysis. For a list of the effects of potentially interfering cellular macromolecules on the assay see Brunk et al., 1979.

Cations, particularly divalent or heavy metal cations cause quenching of the fluorescence. Anions such as citrate, chloride, sulfate and hydrogen phosphate also reduce fluorescence. These phenomena can be summarized as the effect of ionic strength on the fluorescence of the DAPI/DNA complex. Confusion exists over the exact effect of ionic strength on the fluorescence of the DAPI/DNA complex. Brunk et al., 1979 states that at low ionic strengths the fluorescence is decreased. In contrast Kapuscinski and Skoczylas, 1977 state that as the ionic strength increases the fluorescence is decreased. Kapuscinski and Skoczylas, 1977 recommend that the ionic strength of solutions used should be below 0.1. Differing view points about the effect of ionic strength on DAPI/DNA fluorescence are perhaps because different ions are used by different authors or the concentrations of ions used by different authors are widely different. Ionic strength effects are minimized by using a buffer at the same concentration each time an assay is performed.

The fluorescent excitation and emission spectra of DAPI can be seen in figure 10 and also in Kapuscinski and Skoczylas, 1977. Different authors use slightly different excitation and emission wavelengths (Accurate Chemical and Scientific Company, 1988; Leusch et al., 1985; Brunk et al., 1979; Brunk and James, 1977; Joep et al., 1977; Kapuscinski and Skoczylas, 1977; Williamson and Fennell, 1975). Therefore, the excitation and emission wavelength used in this study was taken from measured data (figure 10).

The buffer used in this study was based on Brunk et al., 1979. The buffer used in this study deviated from the Brunk et al., 1979 buffer in that EDTA-disodium-dihydrate was used instead of EDTA. The dihydrate was used because this goes into solution at pH 7 much more easily than EDTA. Trizma base was obtained from the Sigma Chemical Company (P.O. Box 14508, St Louis, MO 63178). The composition of the DAPI/buffer solution used in this study can be seen in table 2. DAPI dissolves relatively easily in water but is more difficult to dissolve in buffer. Therefore, DAPI solution is made up first then the buffer chemicals are added. DAPI.2HCl, the most common form of DAPI, is available as a yellow powder from the Accurate Chemical and Scientific Corporation (28 Tec St., Hicksville, NY 11801).

Table 2: The composition of the DAPI/Buffer solution used in this study.

DAPI/Buffer Component	Concentration
DAPI.2HCl	1 mg/l
NaCl	5.84 g/l
EDTA.2Na.2H ₂ O	3.72 g/l
Trizma Base	1.21 g/l

The assay is temperature sensitive, with fluorescence inversely related to temperature, see figure 11. Constant temperature is therefore very important. Variations in pH between 6 and 9 do not significantly affect the fluorescence of the DAPI/DNA complex, see figure 12.

The fermentation samples containing fishmeal needed to be washed before sonication, otherwise the fluorescence of the DAPI/DNA complex would be attenuated either by the inner filter effect or by quenching effects. This would lead to an underestimate of the cell concentration. Three fishmeal washes were empirically chosen as the best number. The washing of the sample and subsequent half hour centrifugation meant that this assay took a long time to perform. For 12 fermentation samples it took 2 days to obtain the assay results.

To test the interference, different amounts of fishmeal were added to a standard cell solution and the cell concentration was estimated, see figure 13. The effect of fishmeal was negligible over the 0-30 g/l range, which is the range typically used in this study. However, higher concentrations of fishmeal, for example 100 g/l fishmeal, lead to severe interference.

The sonicator used in these experiments was a W350 Sonifier Cell Disruptor (Heat Systems Ultrasonics Inc., 1938 New Highway, Farmingdale, NY 11735). A number 200 one half inch tapped standard disruptor horn was used with a number 406 replaceable flat tip. The sonicator was housed in a super sonabox acoustical enclosure (number 432B). Temperature during sonication was controlled by placing the sample in a stainless steel number 424 cold shoulder which was inside an ice bath. Care of the replaceable flat tips is imperative. Tips should not have corrosion pits in them and if this occurs the tip should be rebuffed or discarded. Corroded tips do not have the same power dissipation characteristics of new tips and often leave a titanium precipitate inside the sample. A new tip was used for each fermentation sample set.

UV grade disposable fluorometric cuvettes type A-204X were obtained from

Spectrocell Inc. (P.O. Box 33, Orelan PA 19075). These cuvettes have a spectral range of 280-800 nm. The fluorescent measurements were obtained on a Perkin Elmer LS-5 fluorescence spectrophotometer (Perkin Elmer, 150 Wells Avenue, Newton Center, MA 02139) and a Perkin Elmer R100A chart recorder. Constant temperature was maintained in a water bath using a B Braun Thermomix 1419 temperature controller.

The fluorescence of the fermentation samples could be related to cell concentration using a standard curve based on solid free samples (see figure 14) or related to a DNA content by using calf thymus DNA as a standard (see figure 15). *Bacillus subtilis* DNA was unavailable. Calf thymus DNA is a good standard, however, because both *Bacillus subtilis* DNA and calf thymus DNA both have an approximate GC content of 42% (Sneath et al., 1986). Serial dilutions are required when analyzing fermentation samples to make sure that the sample is within the linear range of the assay.

Based on calf thymus as a standard *Bacillus subtilis* var *sakainensis* ATCC 21394 had a DNA content of 1.5-2.0%. This compared favorably to a literature value (and calculations of cell volume) of 0.7-1.6% (CRC Handbook of Microbiology, 1981; Powell and Kennedy, 1988).

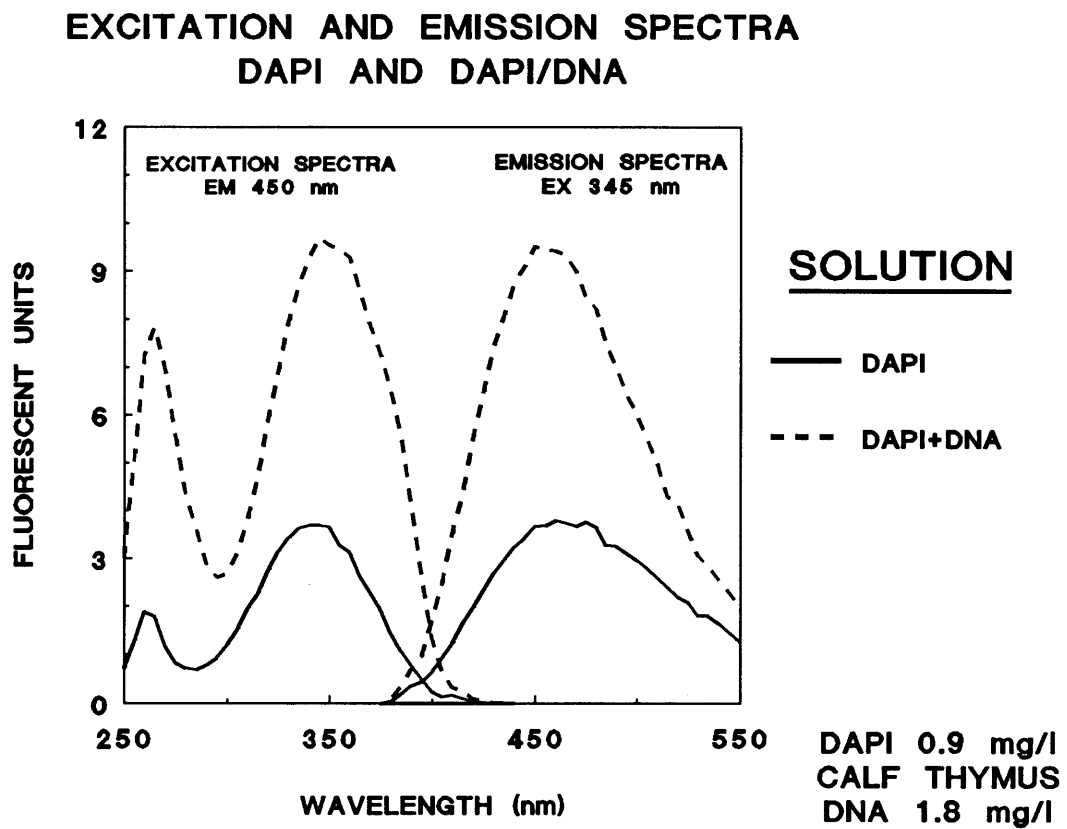


Figure 10: The excitation and emission spectra of DAPI/calf thymus DNA under the conditions used in this study.

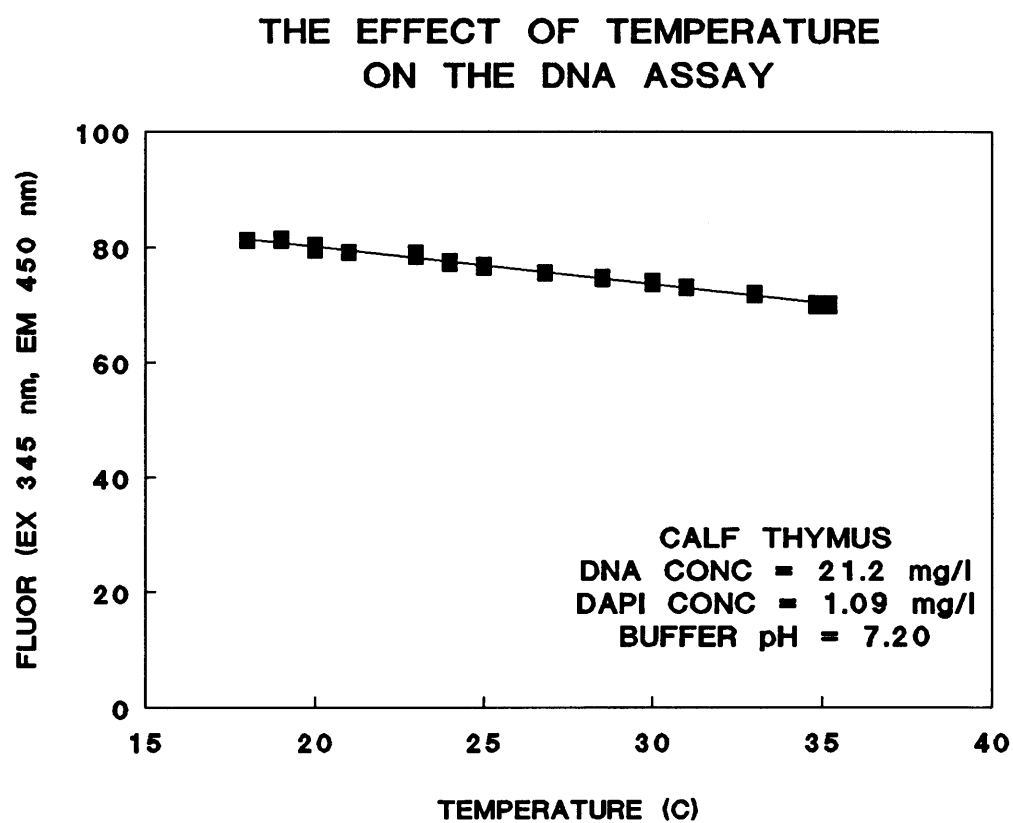


Figure 11: The effect of temperature on the DAPI/calf thymus DNA fluorescent response.

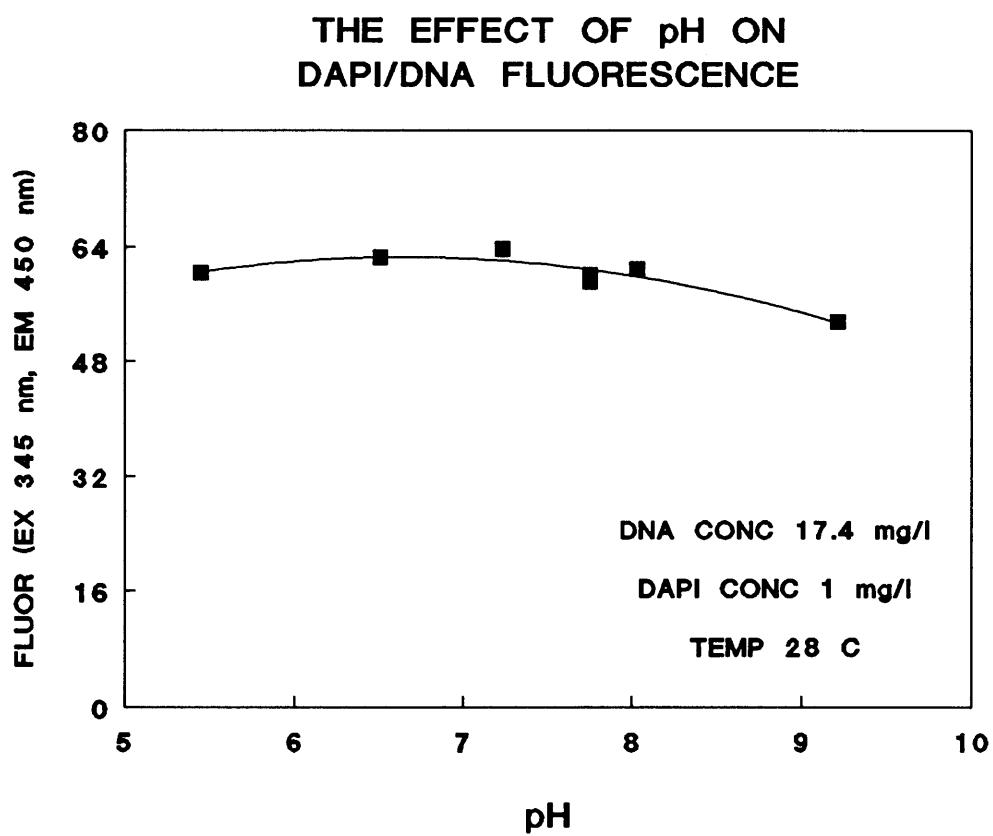


Figure 12: Effect of pH on the DAPI/calf thymus DNA fluorescent response.

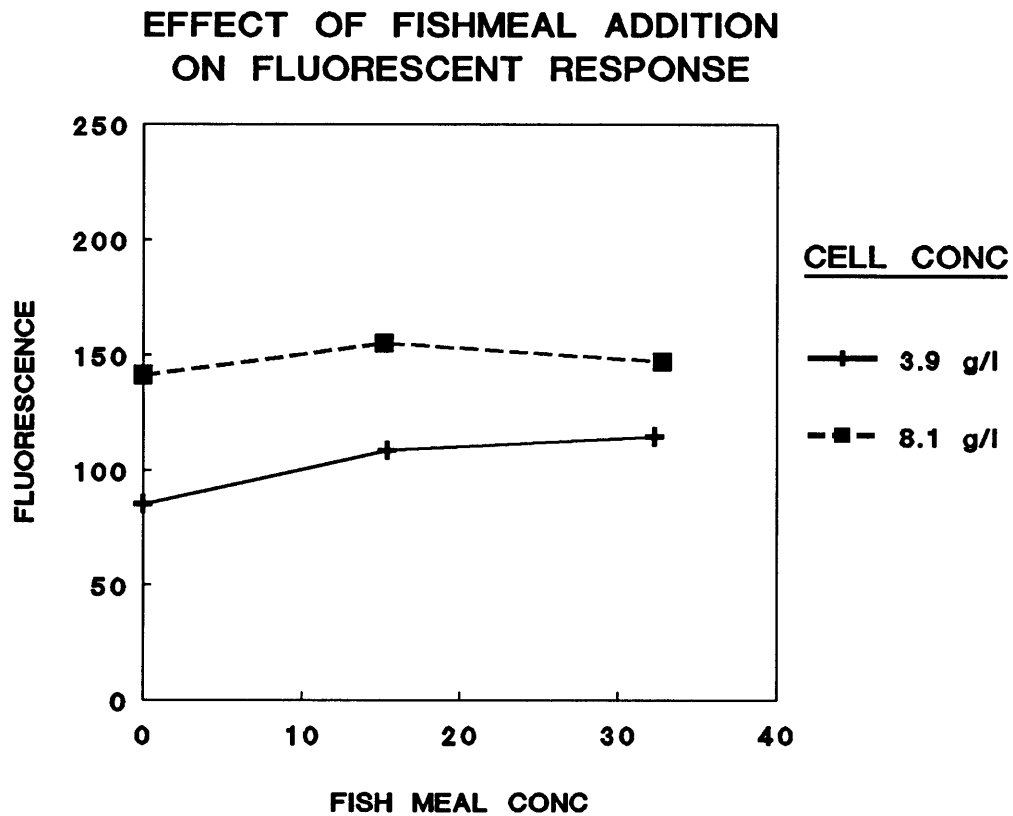


Figure 13: The effect of fishmeal addition on the DAPI/ *Bacillus subtilis* DNA fluorescent response.

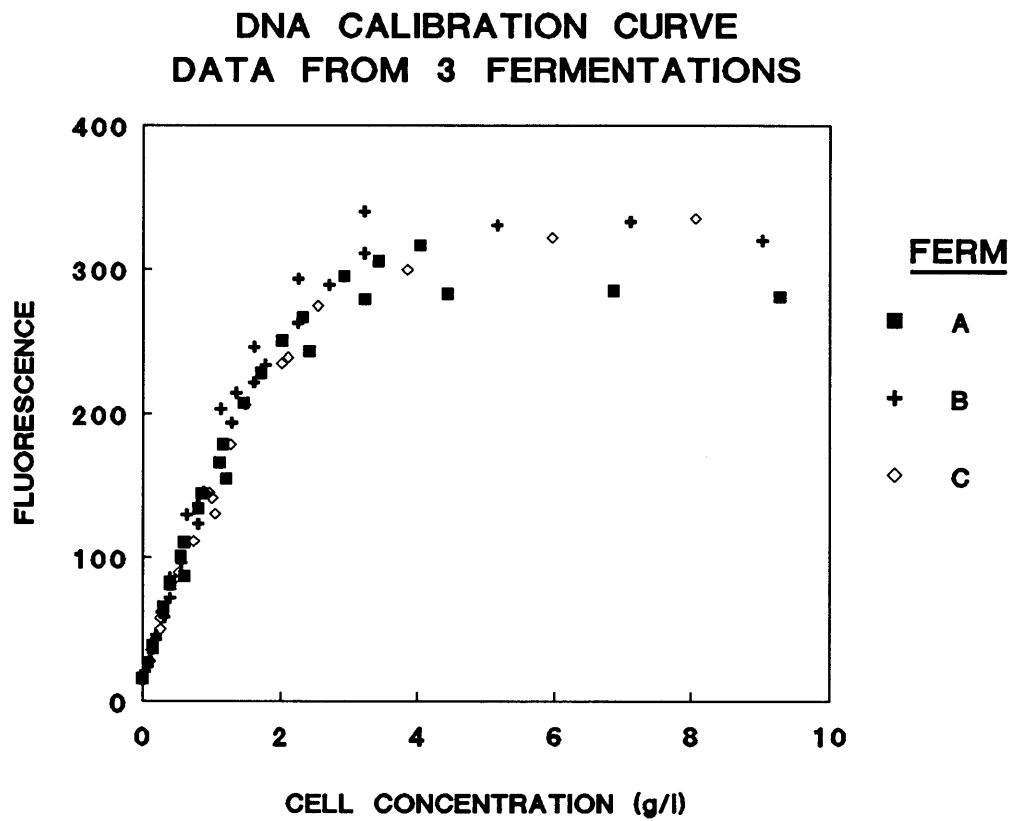


Figure 14: Calibration curve of fluorescence versus *Bacillus subtilis* var *sakainensis* ATCC 21394 concentration. Data from 3 fermentations.

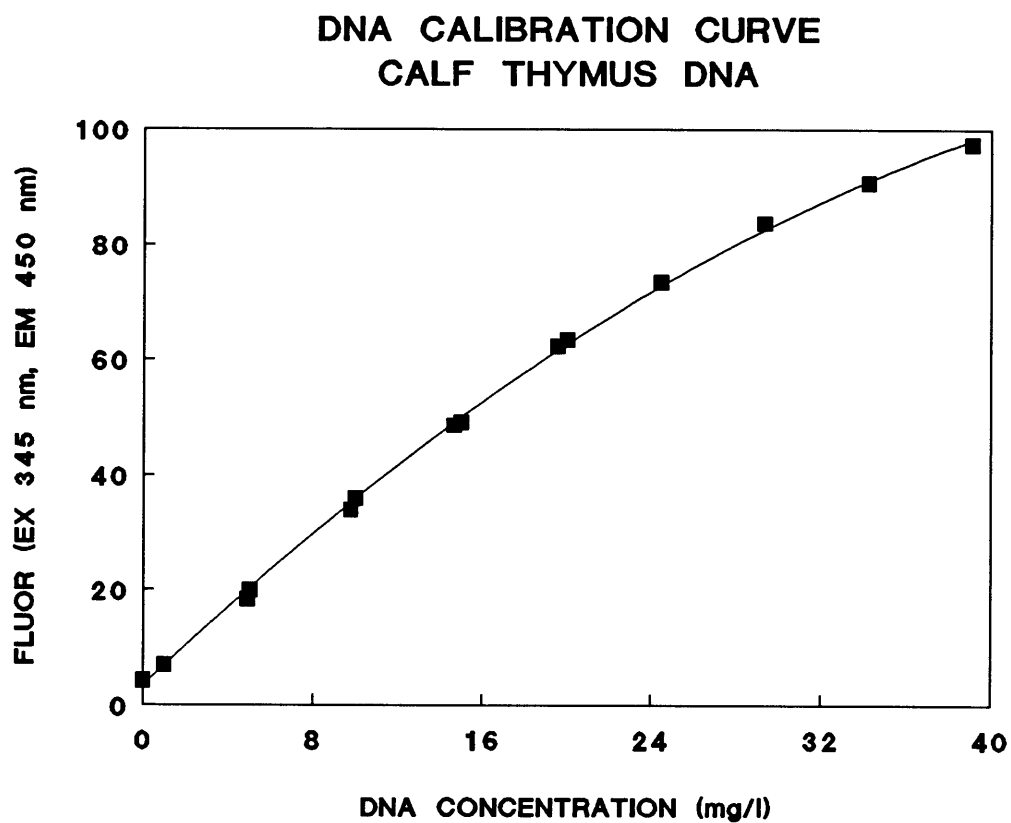


Figure 15: Calibration curve of fluorescence versus calf thymus DNA concentration.

DNA Assay Method

- 1) Make up 2l of a 1 mg/l DAPI.2HCl solution using MilliQ water.
- 2) Add buffer components
- 3) Adjust pH to 7.20 +/- 0.1 pH units.
- 4) Take 10 mls of broth sample.
- 5) Centrifuge (#1) and decant off supernatant. Centrifuge means 10,000 rpm for 30 minutes.
- 6) Wash (#1) with 30 ml of water. Wash means add the water then vortex.
- 7) Centrifuge (#2) and decant off supernatant.
- 8) Wash (#2) with 30 ml of water.
- 9) Centrifuge (#3) and decant off supernatant.
- 10) Wash (#3) with 30 ml of water.
- 11) Centrifuge (#4) and decant off supernatant.

If sample contains fish meal, centrifuge #4 should be 45 minutes at 10,000 rpm to solidify the solids at the bottom of the centrifuge tube otherwise centrifuge should be as usual. Centrifuge #4 should enable an easy and complete separation of solids and supernatant.

- 12) Add 14 ml of DAPI/buffer solution to remaining solids, vortex and transfer to sonication cold shoulder.
- 13) Sonicate with conditions as follows :

Pulsed mode

Time of sonication - 7 minutes

Output of sonicator - 5

% duty of sonicator - 50

Power output - 200 watts

- 14) After sonication, centrifuge (10,000 rpm for 45 min). The centrifuge tubes are balanced by removing liquid from them (ie balance all to the lightest weight).
- 15) Decant and save supernatant, discard residual solids.
- 16) Filter supernatant through 0.22 micron Millex GV filters (Cat No. SLGVO25LS, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730) to ensure that DNA does not bind to the filter.
- 17) Dilute sample with DAPI/buffer solution 1, 4 and 8 times so that you now have 3 samples per original broth sample. Each solution should be at least 4 ml to fill a cuvette.
- 18) Measure fluorescence under the following conditions:
 - temperature of water bath 28.5°C
 - sample temperature equilibration time 30 minutes
 - excitation slit width 5 nm
 - emission slit width 5 nm
 - excitation wavelength 345 nm
 - emission slit width 450 nm
 - fix scale 1.0
 - average 10 readings
- 19) Cells of known concentration are used as a standard. First take a large volume of

broth with no solids present and centrifuge (6,000 rpm for 35 minutes). Decant the supernatant and gather together cell paste.

- 20) Assuming the cell paste to be approximately 230 g/l dcw make up a stock solution of 14 g/l dcw cells. Measure in quadruplicate the dcw of the paste and apply a correction factor to the data later. Approximately 200 mls of 14 g/l stock solution is required. The stock solution is made up by adding 12 g of the 230 g/l cell paste to 200 mls of water.
- 21) Dilute the 14 g/l stock solution in to the following concentrations (g/l):
14, 12, 10, 8, 6, 4, 3, 2, 1, 0.5
- 22) Treat these samples as a normal broth sample as described above except that making the paste counts as centrifuge #1 and making the stock solution and its dilution counts as wash #1. So start with centrifuge (#2) of 10 mls of standardized cell solution.

Disadvantages of the DNA technique.

The use of a DNA technique to estimate cell concentration has one major weakness. The use of the DNA assay assumes that the DNA content of a cell is constant throughout the fermentation. This assumption was not valid in the case of *Bacillus subtilis*. It was found that the DNA content of the cell was a function of growth rate and spore formation activities, see figure 16. Thus the stage of growth of the cells used to calibrate the assay affected the result. This problem was avoided by always calibrating the assay at a fixed low growth rate near the end of the fermentation. This gave the DNA assay the most accuracy

late in the fermentation when the growth rate was low. The assay contained most error early on in the fermentation.

To calculate the amount of error introduced by using the DNA assay, the DNA assay was compared to the dry cell weight analysis during a fermentation which contained no solid substrate (growth on nutrient broth). The results, see figure 17, indicated that, although imprecise, the DNA assay gave an approximate estimate of cell concentration throughout the fermentation. Attempts were made to correct the DNA assay for growth rate but this technique tended to introduce more error and so was discarded.

An advantage of the DNA assay was that it identified the cessation of cell growth and transition to the stationary phase. This advantage made the assay very useful as it complimented the weakness of the carbon dioxide evolution technique. The carbon dioxide evolution technique cannot identify the beginning of the stationary phase.

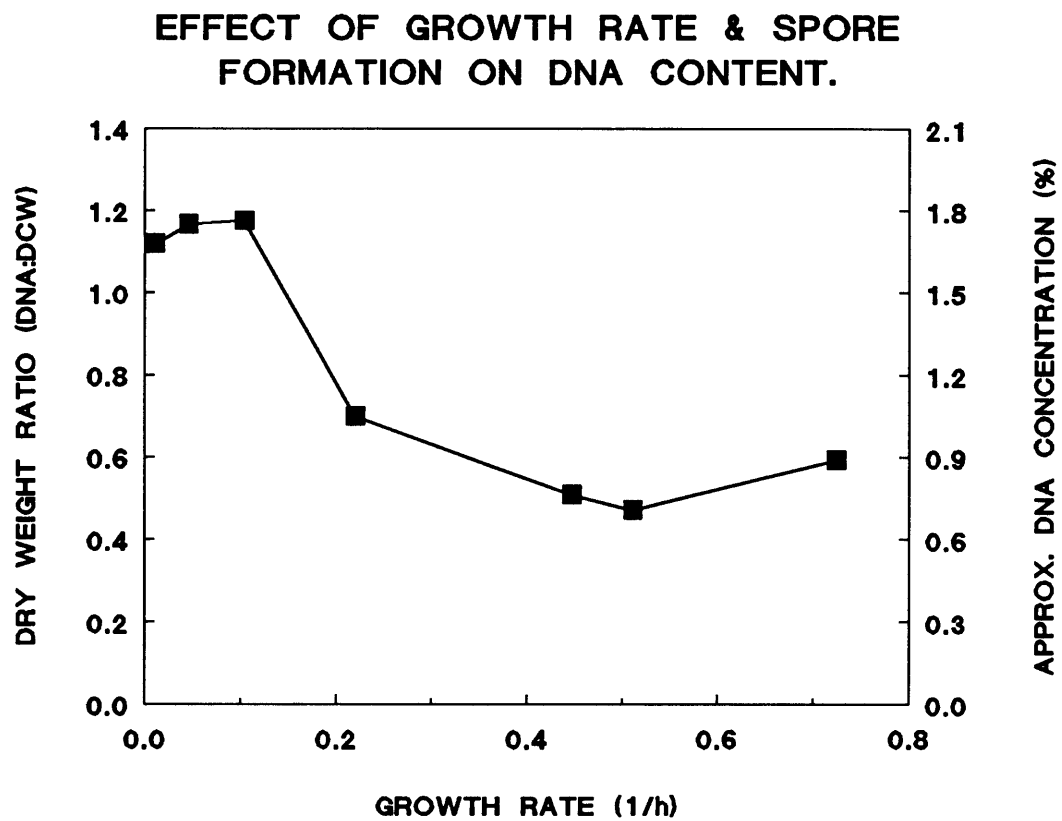


Figure 16: The effect of growth rate and spore formation activities on the DNA content of *Bacillus subtilis* var *sakainensis* ATCC 21394.

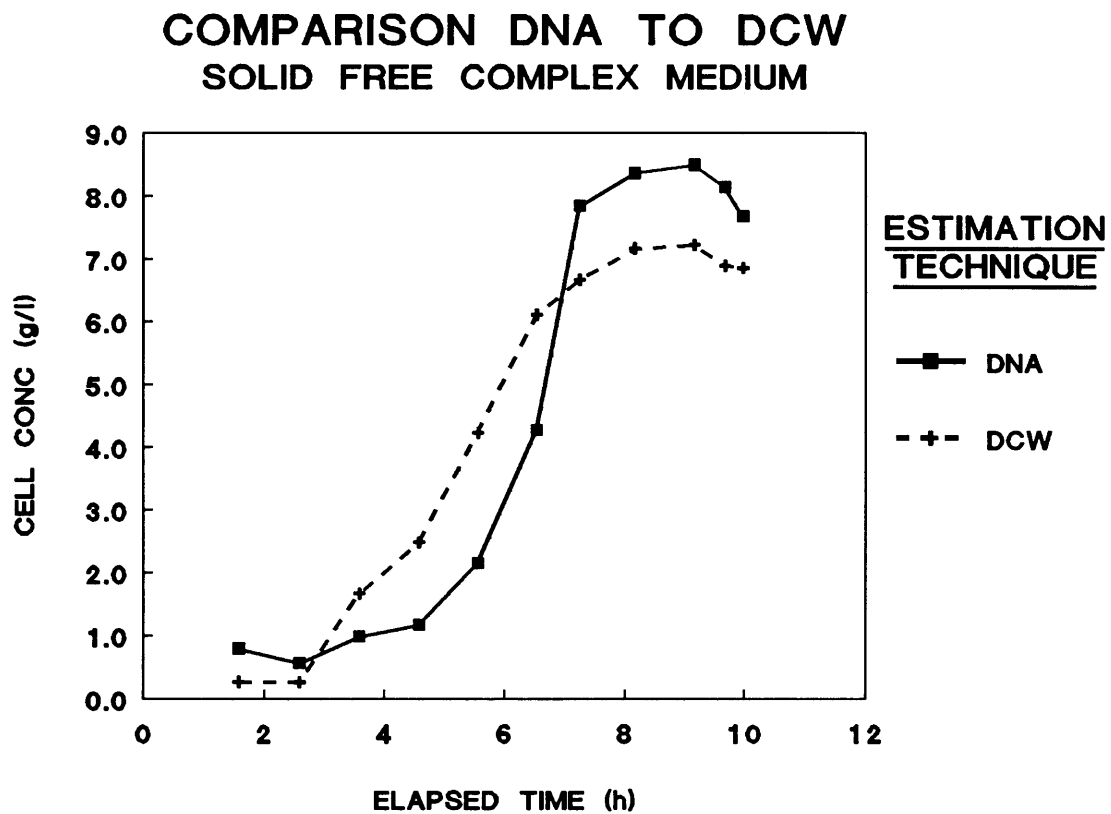


Figure 17: A comparison of the DNA assay and dry cell weight techniques to estimate the cell concentration during the fermentation of *Bacillus subtilis* var *sakainensis* ATCC 21394 on nutrient broth.

3.2.2) Carbon Dioxide Evolution Technique to Estimate Cell Concentration

This technique is based on analyzing the off gas from the fermentor and calculating the total carbon dioxide evolved. The total carbon dioxide evolved is assumed proportional to the accumulated cell concentration. This technique is based on Wang et al., 1979 and has been widely used. The carbon dioxide evolution technique and a similar alternative, based on total oxygen consumed, are popular because the data are available on-line and are not affected by solid substrate being present in the fermentation medium.

The calculation is based on nitrogen as the tie element (one that is not consumed).

First the carbon dioxide evolution rate is calculated as follows:

$$CER = \frac{1}{V_L} \cdot \frac{Q \cdot P}{R \cdot T} \cdot \left(\frac{N_{2,in}}{N_{2,out}} \cdot CO_{2,out} - CO_{2,in} \right) \cdot 60795 \quad (2)$$

where

CER : Carbon dioxide evolution rate (mmoles/l.h)

V_L : Liquid volume in the fermentor (l)

Q : Flowrate of the inlet gas (l/min)

P : Pressure at the fermentor inlet (atm)

R : Universal gas constant (J/mole.K)

T : Temperature of gas at the fermentor inlet (K)

$CO_{2,in}$: Carbon dioxide composition of the inlet gas (%)

$CO_{2,out}$: Carbon dioxide composition of the outlet gas (%)

$N_{2,in}$: Nitrogen composition of the inlet gas (%)

$N_{2,out}$: Nitrogen composition of the outlet gas (%)

The 60795 (101325x60/100) is a factor to make the units consistent.

Next the total carbon dioxide evolved per liter (a unit volume basis was used because in the fed batch fermentations the liquid volume changed during the fermentation) from the beginning of the fermentation was calculated as follows:

$$TOT_{CO_2} = \sum_{t=0}^t CER \cdot \Delta t \quad (3)$$

where:

TOT_{CO_2} : Total amount of carbon dioxide evolved per unit volume (mmoles/l)

t : time (h)

Δt : time interval between scans (h)

Once the total carbon dioxide evolved per liter was known the cell concentration was calculated as follows:

$$X = X_{inoc} + \frac{TOT_{CO_2}}{K_c} \quad (4)$$

where:

X : cell concentration in the fermentor (g/l)

X_{inoc} : cell concentration in the fermentor immediately after inoculation (g/l)

K_c : conversion factor (mmoles CO_2 evolved/g cells accumulated)

The value of K_c was determined by conducting six fermentations on medium containing no solid components and then correlating accumulated dry cell weight with the total carbon dioxide evolved per liter, see figure 18. The value of K_c used in this study was 34.1 mmoles/g, which is within the range 33.6-38.0 mmoles/g normally observed (Wang and Cooney, 1986).

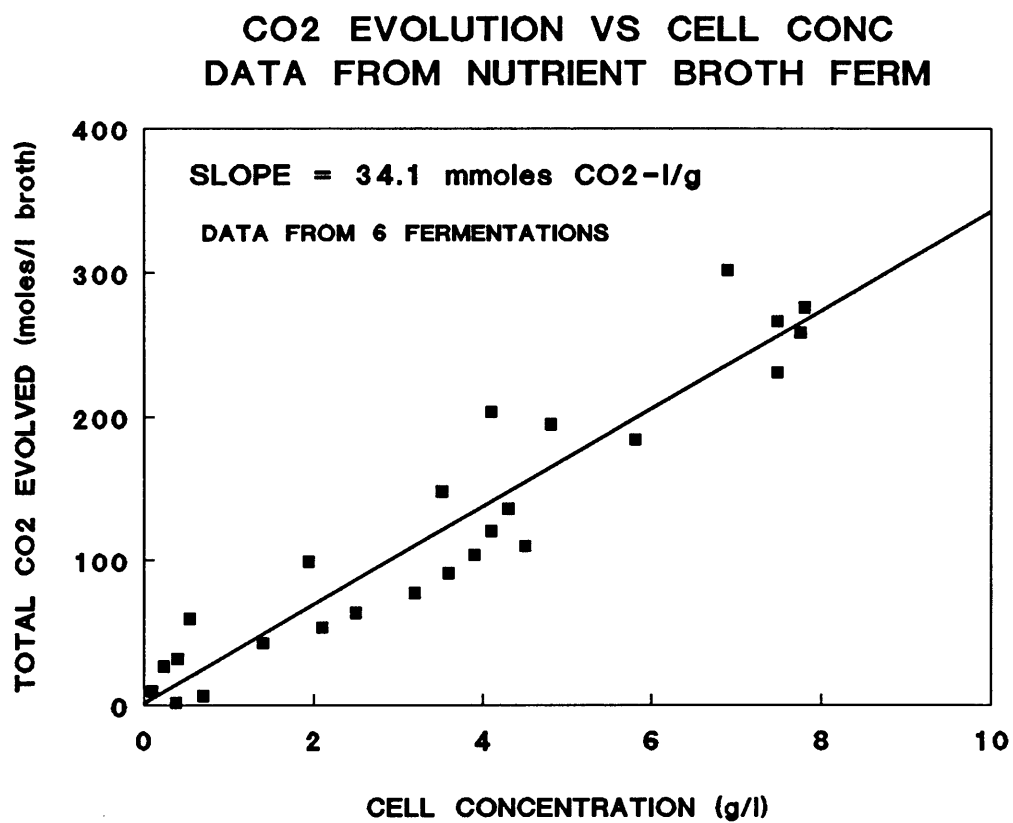


Figure 18: The correlation of dry cell weight accumulated with total carbon dioxide evolved per liter. Data from six fermentations.

Disadvantages of the Carbon Dioxide Evolution Technique

The disadvantage of the carbon dioxide evolution technique is that it fails to predict the cell concentration during the stationary phase of the fermentation. When the culture enters the stationary phase, the cells stop growing but they still produce carbon dioxide. Hence in the stationary phase the cell concentration predicted by the carbon dioxide evolution rate technique continues to rise. The stationary phase is "invisible" to the carbon dioxide evolution technique.

Another disadvantage of the carbon dioxide evolution technique is that the cell concentration in the fermentor immediately after inoculation needs to be estimated. This is often not possible when solid substrate is used in the fermentation medium. Since the cell concentration in the fermentor immediately after inoculation is usually very small, the error introduced into the cell concentration by uncertainties in the initial estimate is usually negligible. This is true at the high cell concentrations near the end of a fermentation. This problem was minimized in this study by preparing the inoculum in a standard way every fermentation and estimating the initial concentration using the DNA technique.

The carbon dioxide evolution technique is most accurate at high growth rates which occur at the beginning of the fermentation. This compliments the DNA assay which is most accurate at low growth rates which occur near the end of the fermentation.

The gas compositions were measured using a mass spectrometer (a model MGA 1200 from Perkin Elmer, 150 Wells Avenue, Newton Center, MA 02159). The mass spectrometer was calibrated periodically with a set of standard gases of known composition. Owing to the distance between the fermentor and the mass spectrometer there was a lag time of

approximately one minute before gas produced by the fermentor would reach the mass spectrometer. In addition to this lag time, the mass spectrometer read the gas composition every 60 seconds with a hold time of 20 seconds. Paragon scanned the mass spectrometer for data every 2 minutes. Thus the time between the gas being produced by the cells and the cell concentration being estimated was approximately 3-4 minutes. This 3-4 minute delay time was insignificant compared to the time constant of the fermentation which is of the order of 1 hour.

3.2.3) Fishmeal Assay

The fishmeal concentration was determined using a dry weight measurement and a cell concentration estimate based on either light scatter, DNA, or carbon dioxide evolution techniques. The fishmeal concentration was obtained by subtracting the cell concentration from the dry weight.

Dry Weight Assay

- 1) Vortex the sample in its initial container.
- 2) Transfer entire sample to beaker and stir.
- 3) Using a 5 ml repeater pipette (the end is cut off the plastic pipette tip to enlarge the hole so a representative sample is taken) transfer 25 ml into a 25 ml volumetric flask.
- 4) Set liquid level to the mark of the volumetric flask.
- 5) Transfer from flask to centrifuge tube.
- 6) Rinse out the volumetric flask into the centrifuge tube.
- 7) Balance centrifuge tubes by adding distilled water.
- 8) Spin for 30 minutes at 10,000 rpm.
- 9) Decant the centrifuge tubes.
- 10) Wash the residue (add 30 ml with the 5 ml repeater pipette and vortex).
- 11) Balance centrifuge tubes by adding distilled water.
- 12) Spin for 30 minutes at 10,000 rpm.
- 13) Decant the centrifuge tubes.
- 14) Weigh out the dry weight aluminum tins

- 15) Transfer the residue from the centrifuge tubes into the corresponding dry weight tins.
Rinse out the centrifuge tubes with a small amount of water into the dry weight tins.
- 16) Place tins in the dry weight oven (60°C) for approx 48 h.
- 17) Remove tins from oven and place in the desiccator for 30 minutes.
- 18) Weight the tins & contents.

Disadvantages of the Fishmeal Assay

The main disadvantage of the fishmeal assay is that any error in estimating the cell concentration is propagated into the fishmeal concentration.

3.2.4) Glucose, Acetate and Acetoin Assays

Glucose, acetate and acetoin concentrations were determined using an HPLC system. The HPLC system consisted of a WISP 701B autosampler, a R401 differential refractometer, M6000A pump, all from Waters Chromatography Division (34 Maple St., Milford, MA 01757), an organic acid column HPX-87H from Bio-Rad Laboratories (3300 Regatta Blvd., Richmond, CA) and a HP3390A Integrator from Hewlett-Packard (Eastern Sales Region, 29 Burlington Mall Road, Burlington, MA 01803). The column was kept at 40°C using a Biorad column heater. A sulfuric acid buffer (0.56ml of concentrated sulfuric acid per 2 liters of deionized water) was pumped at 0.5 ml/min throughout the operation of the HPLC. Fermentation broth samples were filtered through 0.22 micron Millex GV filter (catalog number SLGVO25LS, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730) prior to HPLC analysis. The injection volume used was 40 μ l.

Using this setup glucose, acetate and acetoin had residence times of approximately 11.3, 19.0, and 22.3 minutes respectively. The integrator program used can be seen in figure 19, typical calibration curves can be seen in figures 20 and a typical spectrum obtained during the fermentation can be seen in figure 21. Glucose, acetate and acetoin were identified by comparing the residence time of the peaks in the fermentation broth with those of the pure compounds. Complete identification involving the addition of the pure compound to the fermentation sample, testing for an increased peak height and the absence of shoulders was not conducted.

The HPLC buffer had a pH of approximately 2 so any acetate in the fermentation broth sample ($pK_a=4.7$) would immediately transfer to the undissociated form when

introduced into the HPLC. Thus the HPLC detected acetic acid, whereas at the pH of the fermentation (pH 7) most of the acid would be in the dissociated form (acetate). All numbers for acetate reported in this study are representative of the acetate concentration at pH 7 but are in acetic acid equivalents as this is all that the HPLC could measure.

Glucose concentration was correlated with peak area or peak height. Acetate and acetoin concentrations were correlated with peak height. Peak height was used because other compounds in the fermentation broth had very similar residence times to acetate and acetoin and sometimes the peaks would overlap to a small extent. For example, acetoin has a residence time of 22.3 minutes and 2,3-butanediol, also produced during this fermentation, had a residence time of 23.2 minutes. Peak overlap between these two compounds often occurred and hence an analysis based on peak area would have given incorrect results.

Disadvantages of the HPLC technique

The HPLC technique took 30 minutes to run which meant a considerable lag time between taking a sample and getting a result to take corrective action, for example, in correcting the glucose control strategy. Often HPLC analysis was done after the fermentation was complete.

RUN PARAMETERS

ZERO = 10
ATT 2↑ = 6
CHT SP = 0.2
PK WD = 0.64
THRSH = 6
AR REJ = 1000

RPRT OPTNS

2. RF UNC PKS = 0.0000E+00
3. MUL FACTOR = 1.0000E+00
4. PK HEIGHT MODE NO
5. EXTEND RT NO
6. RPRT UNC PKS YES

TIME TBL

0.00 ZERO = 10
0.00 ATT 2↑ = 6
0.00 INTG # = 4
0.00 INTG # = -7
0.00 INTG # = -8
0.00 INTG # = -9
0.00 PK WD = 0.64
30.00 STOP

CALIB
EMPTY

Figure 19: The integrator program used to calculate HPLC results.

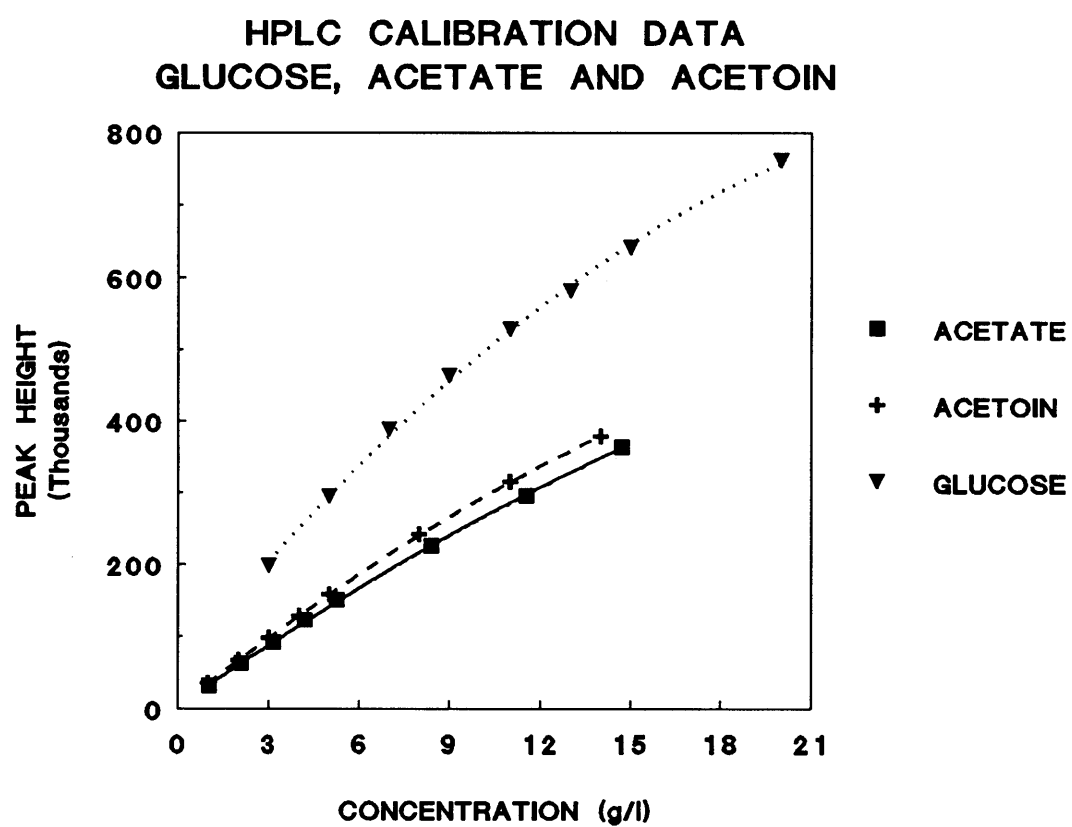


Figure 20: Calibration curves for glucose, acetate and acetoin.

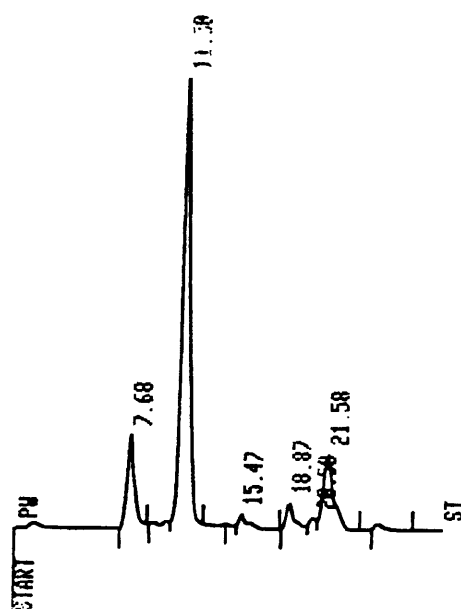


Figure 21: A HPLC spectrum obtained from the fermentation of *Bacillus subtilis* on fishmeal.

3.2.5) Protease Assay

Protease activity is measured by digesting a suitable protein substrate with the sample for a given length of time, then measuring the formation of some product of the substrate degradation. There are three common methods for measuring protease activity. The Kunitz or casein digestion method (Kunitz, 1947) uses casein as the protease substrate and measures tyrosine and other amino acids by absorbance at 280 nm. The Anson or hemoglobin method (Anson, 1938) uses hemoglobin as the protease substrate and measures tyrosine and other amino acids by absorbance at 280 nm. The azocasein technique (Charney and Tomarelli, 1947) uses a chromogenic substrate, azocasein. The azo group which is cleaved from the substrate during digestion is monitored by measuring absorbance at 440 nm.

Numerous other techniques have been described in the literature that use either very specific substrates or specially designed polypeptides as the substrate. Fluorometric techniques can also be used to identify degradation products.

The azocasein technique was chosen for this study because:

- 1) This assay has been used numerous times for estimating protease concentration during the fermentation of *Bacillus subtilis*.
- 2) Protease fermentation of solid meals leads to the formation of amino acids in the fermentation broth. The azocasein assay was chosen to avoid potential interference by large amounts of amino acids on the Kunitz or Anson techniques.
- 3) The absorbance of broth samples is highest at low wavelengths. The azocasein technique was chosen because it measures absorbance at a higher wavelength than

the Kunitz or Anson techniques and hence broth sample absorbance is less of a problem.

- 4) The azocasein substrate is a large protein molecule of similar size to proteins encountered in solid meals. Small polypeptides substrates were avoided as it was thought that protease activity on small polypeptides would be different to that on solid meals.

The following technique was adapted from Rahaman et al., 1988.

Method for alkaline protease activity

- 1) The fermentation broth sample was first centrifuged and the cells and solid substrate removed. The supernatant was diluted 1:9 (ie 10 times)
- 2) 0.4 ml of 2%wt azocasein (catalog number A-2765, Sigma Chemical Company, P.O. Box 14508, St Louis, MO 63178) in phosphate buffer pH 11 is added to 0.4 ml of the diluted sample. The pH of the buffered azocasein solution was checked on a pH meter before use.

Phosphate buffer pH 11 (Bates and Bower, 1956)

50 ml of 7.10 g/l Na_2HPO_4 + 4.1 ml 0.1M NaOH diluted to 100 ml with H_2O .

- 2) The sample is allowed to digest at 34°C (temperature of the fermentation) for 1 h. Samples are started (and stopped) digesting at 15 second intervals.
- 3) 2 ml of 5% trichloroacetic acid is added to stop the reaction after 1 h.
- 4) The samples are kept at 34°C for a further 10 minutes to stabilize the color.

- 5) The samples are then filtered through 0.22 micron Millex GV filters (non-absorbing SLGVO25LS, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730).
- 6) 1 ml of the supernatant is then added to 1 ml of 2N NaOH.
- 7) Also 0.5 ml of the supernatant is then added to 1 ml of 2N NaOH. This is to provide a reading if the sample is very concentrated and also to confirm by serial dilution that the assay is in the linear range. 0.5 ml of the supernatant is added to 2 ml of 2N NaOH if further dilution is required.
- 8) The samples are then read at 450 nm on the Vmax microplate reader. The sample volume in plate well was 0.2 ml.

Blanks

The purpose of doing a blank is to correct for the optical density (450 nm) of the sample. All final absorbance readings (and hence activity) are reported as a sample reading minus a blank reading from the same sample.

- 1) 2 ml of 5% trichloroacetic acid is added to 0.4 ml of sample.
- 2) After trichloroacetic acid addition 0.4 ml of azocasein is added.
- 3) The mixture is digested at 34°C for 1 h.
- 4) The mixture is left for 10 minutes at 34°C to stabilize the color.
- 5) The sample is then filtered through a 0.22 micron filter Millex GV filter.
- 6) 1 ml of the supernatant is then added to 1 ml of 2N NaOH.
- 7) Also 0.5 ml of the supernatant is then added to 1 ml of 2N NaOH. This is to provide a reading if the sample is very concentrated and also to confirm by serial

dilution that the assay is in the linear range. 0.5 ml of the supernatant is added to 2 ml of 2N NaOH if further dilution is required.

- 8) The samples are then read at 450 nm on the microplate reader (0.2 ml of sample per plate well).

Method for neutral protease activity

The method for determining neutral protease is identical to that for alkaline protease except that a pH 7 phosphate buffer is used.

Phosphate buffer pH 7 (Gomori, 1955).

39 ml of 27.8 g/l NaH_2PO_4 + 61 ml of 53.65 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ diluted to 200 ml with H_2O .

Equipment

Optical density was read on the "Vmax", kinetic microplate reader and data logging was performed using the "SOFTmax" software package version 1.02 (Molecular Devices Corporation, 3180 Porter Drive, Palo Alto, CA 94304).

Reported Protease Units

Two units of protease activity are reported in this study, alkaline protease units (APU) and neutral protease units (NPU). The definition of protease activity reported in this study was based on the standard acid protease assay definition, (Barfoed, 1983, p 557).

One alkaline protease unit (APU) is that amount of enzyme that will produce a change of absorbance of 0.1 at 450 nm under the conditions of the assay (digestion at pH 11).

One neutral protease unit (NPU) is that amount of enzyme that will produce a change of absorbance of 0.1 at 450 nm under the conditions of the assay (digestion at pH 7).

Note that the above definitions do not measure alkaline and neutral concentrations but rather measure total activity at pH 7 and pH 11 respectively. Also note that the fermentation broth was centrifuged and the solid substrate and cells removed before the assay began. This means that the reported protease activity was on a unit liquid volume basis not on a unit total volume basis. This basis was chosen because it gave a clearer indication of the protease concentration available for recovery and purification after solid removal.

3.2.6) Light Scatter Spectrum Measurement Technique

The light scatter spectrum of a sample was measured as follows. One hundred ml of the sample was placed in a glass beaker along with a small magnetic stir bar. The beaker was painted black on the outside to prevent any stray light from affecting the reading. The front face probe was then placed in the beaker with the tip of the probe submerged to a depth of 1 cm. The probe was submerged only to a depth of 1 cm because in very dilute solutions or samples of only water, scatter from the base of the beaker was measured if the probe was submerged to a greater extent. In the typically optically dense solutions obtained from fermentations the path length or penetration distance was very small, on the order of 1 mm, so the depth of submersion did not affect the readings. However, in all samples the probe was submerged 1 cm to be consistent.

The entire beaker plus the probe was wrapped in tin foil to prevent stray light from entering the system. The wrapped assembly was placed on a magnetic stirrer and the sample agitated vigorously. This was to suspend the solid substrate and cell particles during the measurement so that the probe saw a representative sample. Vortex formation was avoided by four small baffles made of 1/4 inch stainless steel tube affixed to the inside of the beaker. A photograph of the wrapped assembly can be seen in figure 22.

Painting the beaker black and covering the assembly with tin foil was more a precaution than a necessity as most of the samples were so optically dense that no outside light reached the probe when it was submerged in the sample. However, with very dilute samples, say less than 1 g/l cells and less than 10 g/l fishmeal, some shielding may be required.

The light scatter spectrum was then obtained by taking a light scatter reading every 10 nm between 330 and 700 nm using the Fluorescence Spectroscopy v 1.1 program run on an Apple IIE computer. Each light scatter reading was comprised the average of a series of readings taken over a one second interval. A complete spectrum between 330 and 700 nm took 3 minutes 20 seconds to generate.

The standards used to calibrate the light scatter probe consisted of a known concentrations of cells and/or fishmeal. The cell concentrations were made up as follows. *Bacillus subtilis* var *sakainensis* ATCC 21394 was grown in medium containing no solid substrate, see section 3.4.2. After a period of growth the cells were harvested and left to sediment overnight at 4°C. The cell sediment was then centrifuged to obtain a cell paste at a concentration of approximately 230 g/l. The concentration of the cell paste was later accurately determined by drying a known weight of paste for 48 h at 60°C then weighing the residue. The cell paste was then diluted with water (not fermentation medium) to give the desired cell concentration.

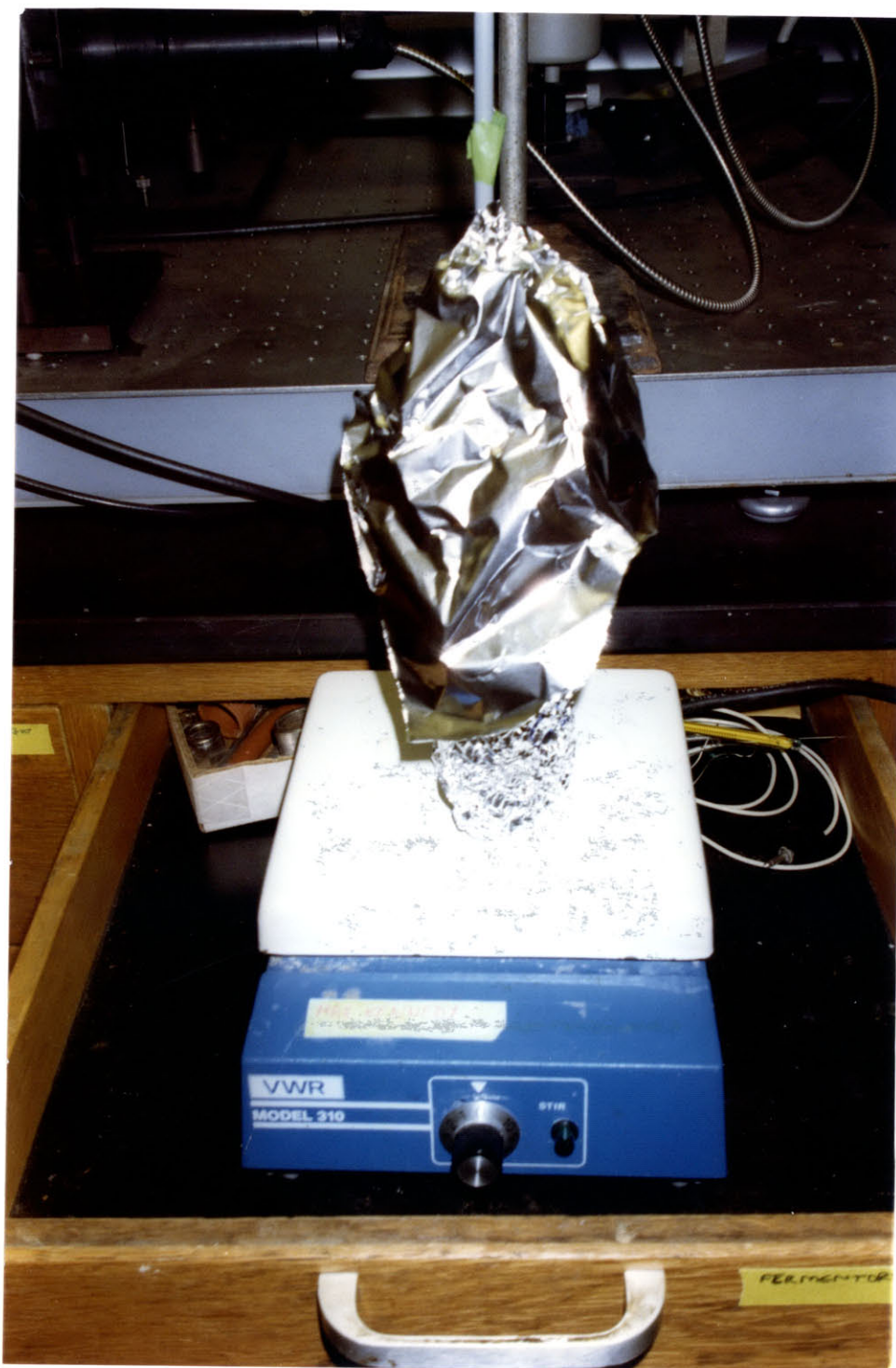


Figure 22: The wrapped sample assembly used to measure the light scatter spectrum.

3.3) Equipment Setup and Control Strategies

3.3.1) Equipment Setup

A schematic diagram of the experimental setup can be seen in figure 23. Photographs of the various components can be seen in figures 24, 25, and 26. Computer number 2 was the main control computer and ran the overall Paragon program. It was connected to Computer number 1 which recorded data from the mass spectrometer. Light scatter data and HPLC data was manually entered into the Paragon control program at computer number 2. The Opto 22 signal conditioning unit collected data from the fermentor and controlled the mass flow controllers and the glucose addition pump.

The balances initially provided a problem because they all had RS-232 connectors on them but there was only one serial port available on computer number 2 to accept the 3 inputs. Hence a multiplexer was set up to enable one serial port to read 3 balance inputs. The multiplexer used was a model ASO-2205 (Ohaus Scale Corporation, 29 Hanover Road, Florham Park, New Jersey 07932). The C routine used to address the multiplexer can be seen in appendix B. Balance 1 under the fermentor (to estimate fermentation volume) was a LB-60 (28kg x 10g), balance 2 under the NaOH feed was a GT2100 (2kg x 0.01g), balance 3 under the glucose feed was a GT4000 (4kg x 0.1g), and balance 4 under the fishmeal feed was a GT4000 (4kg x 0.1g), all from Ohaus Scale Corporation. Balances 1,2, and 3 were connected to the multiplexer and hence Paragon but balance 4 was operated off-line and the results manually entered into Paragon.

When a sample was taken, it was analysed on the HPLC, a light scatter spectrum was obtained, photographs were taken at 1000x magnification under oil and a part of the sample

stored for later analysis of DNA, protease and dry weight. Scanning electron microscope photographs were taken of samples from different stages of one particular fermentation.

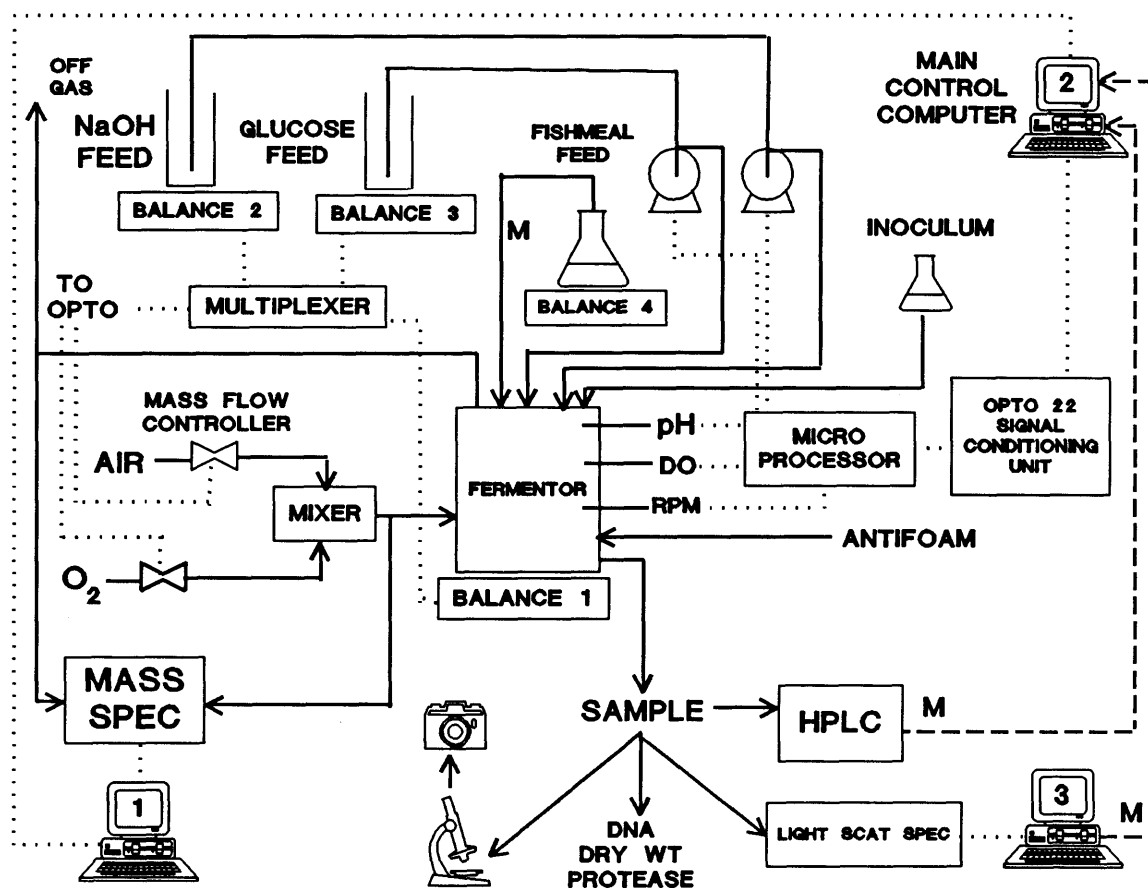


Figure 23: A schematic diagram of the experimental setup used in this study. M signifies that data was manually entered.



Figure 24: The overall experimental setup showing the fermentor, feed vessels, multiplexer, mass flow controller and Opto 22 signal conditioning unit.

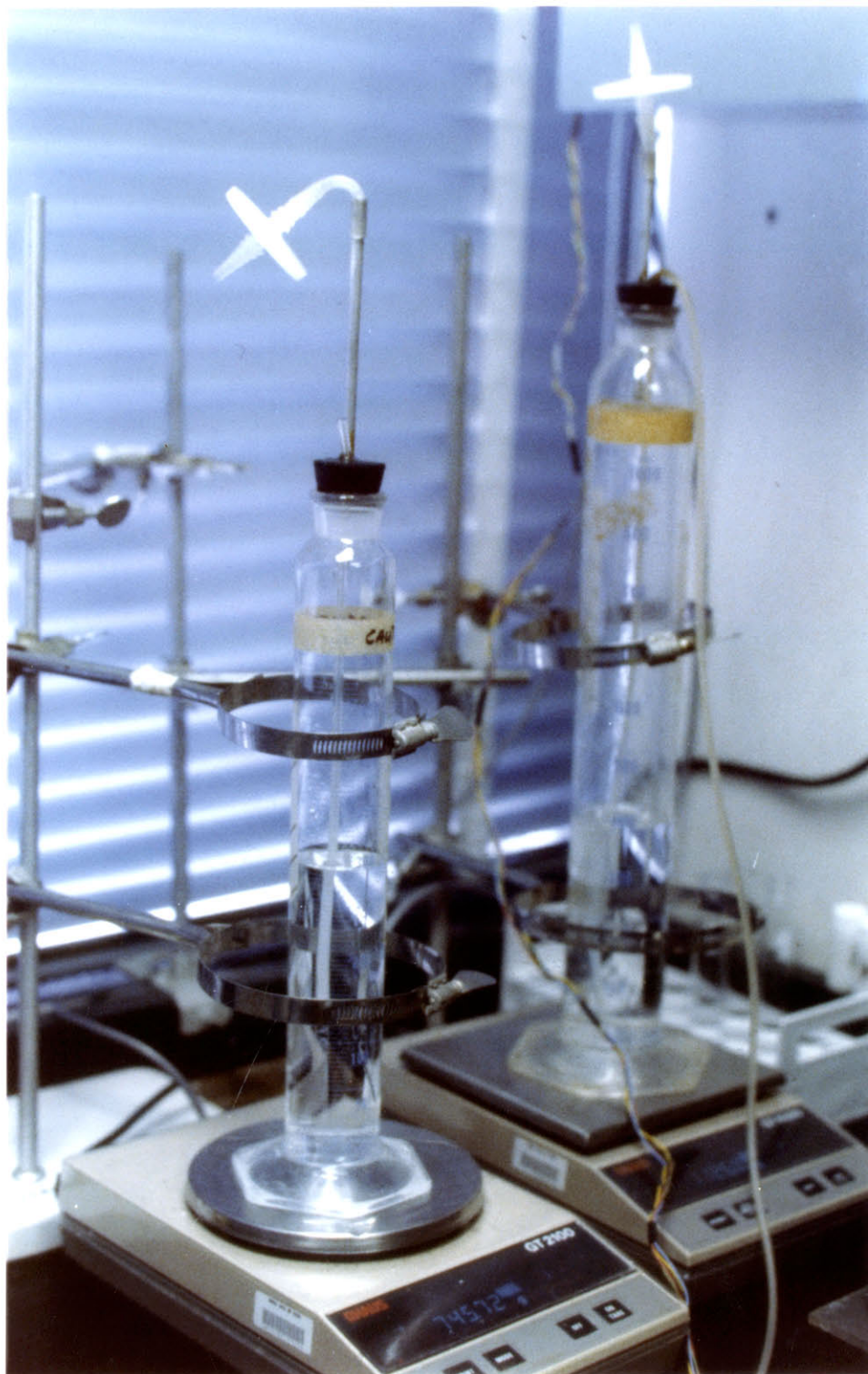


Figure 25: Detail showing the glucose and NaOH feed vessels and balances.

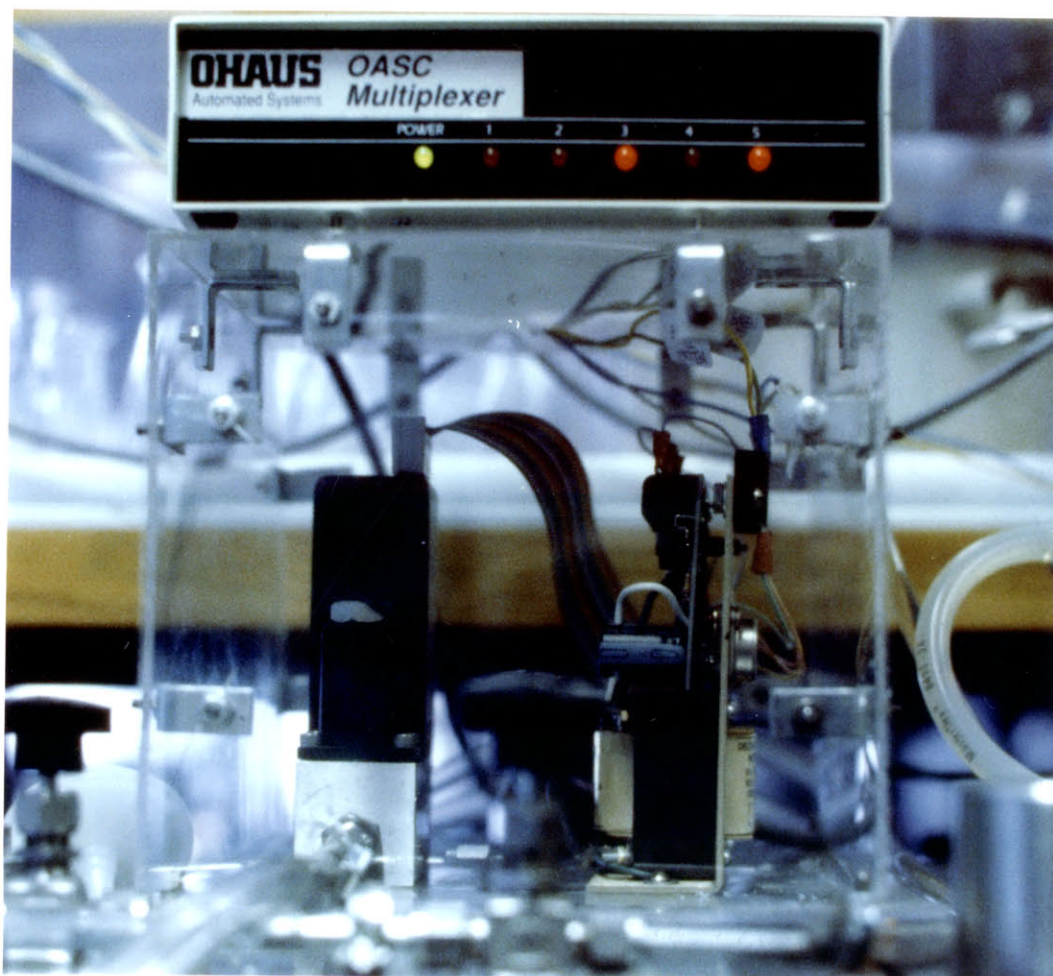


Figure 26: Detail showing the multiplexer and a mass flow controller.

3.3.2) Glucose Control Strategy

Glucose was controlled at approximately 9 g/l during most of the fermentations for three reasons:

- 1) Large quantities of glucose (> 40 g/l) added batchwise at the beginning of the fermentation would inhibit the growth of the cells.
- 2) In this study the effect of the solid substrate was of most interest, so the effect of glucose was minimized by keeping it approximately constant.
- 3) Glucose is known to repress the formation of proteases, so keeping glucose at a low level is desirable.

Glucose was controlled based on carbon dioxide evolution. Paragon was used to perform an on-line mass balance on glucose as follows:

$$\begin{array}{ccccccc} \textit{Glucose in} & & \textit{Glucose} & \textit{Glucose removed} & \textit{Glucose initially} & \textit{Glucose consumed} & \\ \textit{fermentor} & - & \textit{added} & - & \textit{in samples} & + & \textit{in medium} & - & \textit{by the cells} & \\ (g) & & (g) & & (g) & & (g) & & (g) & \end{array} \quad (5)$$

Once the amount of glucose in the fermentor was known the glucose concentration could be calculated because the fermentation volume was known from balance 1. The total amount of glucose added was determined using data from balance 3 under the glucose feed reservoir and the glucose concentration and the density of the feed. The amount of glucose removed in all samples was determined as follows:

$$G_S = \sum_{n=1}^N G_f \cdot V_S \quad (6)$$

where:

G_S : Glucose removed in all the samples (g)

N : Total number of samples taken

G_f : Estimated glucose concentration in the fermentor at the time of the sample (g/l)

V_S : Volume of the sample (l)

The volume of each sample was determined on-line by Paragon as follows:

$$V_S = \frac{1}{\rho_f} \cdot [G_A + S_A + FM_A - \Delta F_w] \quad (7)$$

where:

G_A : Weight of glucose solution added since the last scan (g)

S_A : Weight of sodium hydroxide solution added since the last scan (g)

FM_A : Weight of fishmeal added since the last scan (g)

ΔF_w : Change in weight of fermentor since the last scan (increase in weight = +ve) (g)

ρ_f : Density of the fermentation broth (g/l)

G_A is determined using data from balance 3. S_A is determined using data from balance 2. FM_A was calculated off-line using balance 4 and manually entered into Paragon. ΔF_w was determined using data from balance 1. Often S_A was small and could be neglected.

The glucose initially present in the medium was determined from the medium composition and manually entered into Paragon. The glucose consumed by the cells was based on carbon dioxide evolution as follows:

where:

G_{con} : Glucose consumed (g)

$$G_{con} = \frac{TOT_{CO_2}}{K_A} \quad (8)$$

TOT_{CO_2} : Total amount of carbon dioxide evolved per unit volume (mmoles CO_2 evolved/l), see equation 3.

K_A : Conversion factor (mmoles CO_2 evolved/g glucose consumed)

The constant K_A is comprised of two other constants as follows:

$$K_A = K_C \cdot Y_{X/S} \quad (9)$$

where:

K_C : Conversion factor (mmoles CO_2 evolved/g cells accumulated)

$Y_{X/S}$: Yield coefficient (g glucose consumed/g cells accumulated)

The coefficients K_C and K_A are experimentally determined, see figures 18 and 27.

The values obtained in the study are as follows.

K_C : 34.1 mmoles/g

K_A : 16.94 mmoles/g (fishmeal as nitrogen source)

11.33 mmoles/g (nutrient broth as nitrogen source)

The ability of the glucose control strategy to predict glucose concentration can be seen in figures 28 and 29. To keep glucose constant the glucose control pump was turned on as soon as the predicted glucose concentration fell below the desired set point.

Disadvantages of the Glucose Control Strategy

There are two disadvantages to using carbon dioxide evolution to control glucose. The most significant problem is that $Y_{x/s}$ (and hence K_A) is not constant throughout the entire fermentation. When the growth starts to slow down and the stationary phase is approached these constants will typically change. Estimating these changes can be a trial and error procedure.

The second problem in using carbon dioxide evolution to control glucose is that the amount of glucose initially present in the medium needs to be estimated. This number can be estimated from the initial medium composition but some glucose also comes in from the inoculum. It is often difficult to estimate the amount of glucose in the inoculum. If very tight control of glucose is required then this may be a problem.

To overcome these difficulties the HPLC system was used to estimate the glucose concentration in each sample that was taken. When the results were available (30-40 minutes after the sample was taken) the glucose estimate based on carbon dioxide evolution was corrected to reflect the accurate HPLC measurement. This HPLC correction system was not used in all fermentations.

Using the HPLC to correct glucose estimates did not lead to perfect glucose control because of other factors such as problems with Paragon and the glucose feed system. Such problems were always correctable but difficult to correct on-line during the fermentation.

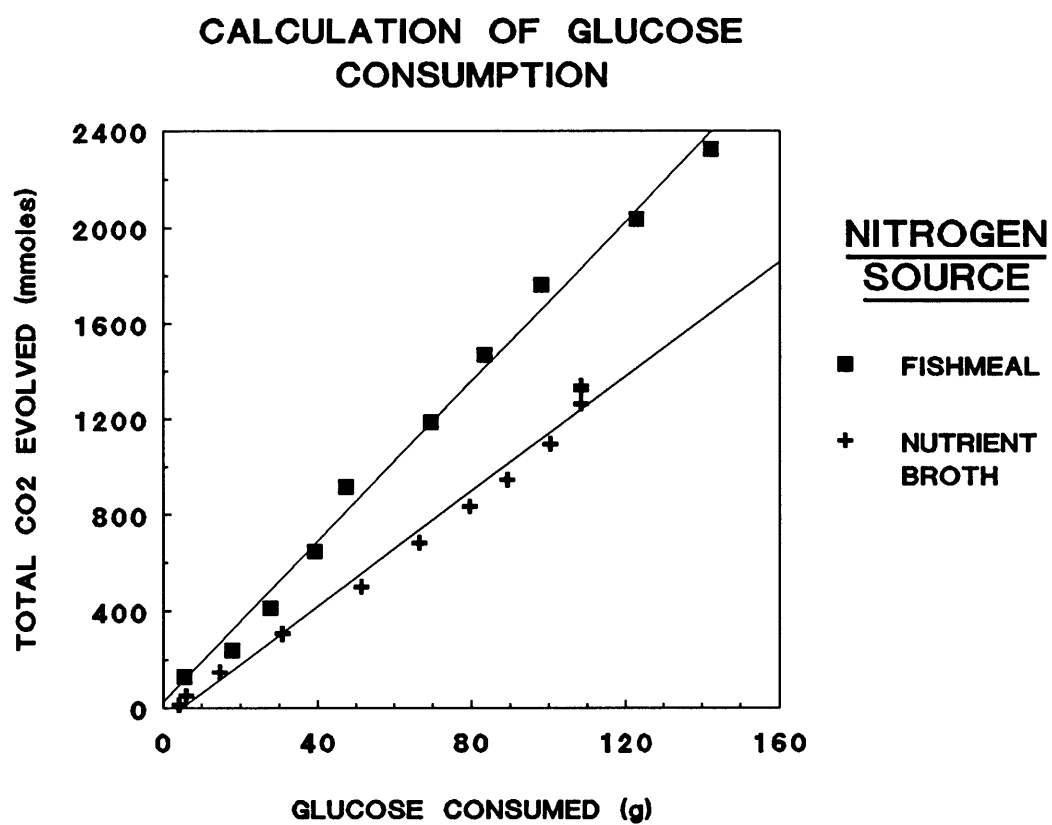


Figure 27: The calculation of K_A for fishmeal and nutrient broth as nitrogen sources.

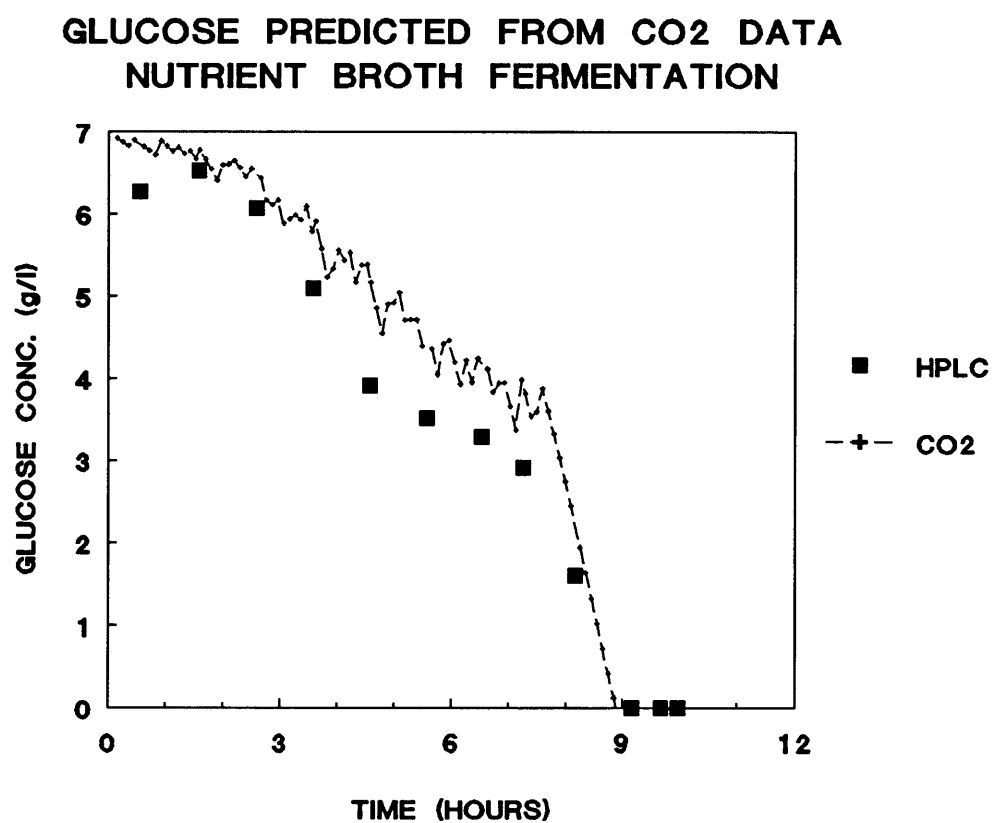


Figure 28: Prediction of the glucose concentration in a nutrient broth fermentation using the K_A value calculated from the same data.

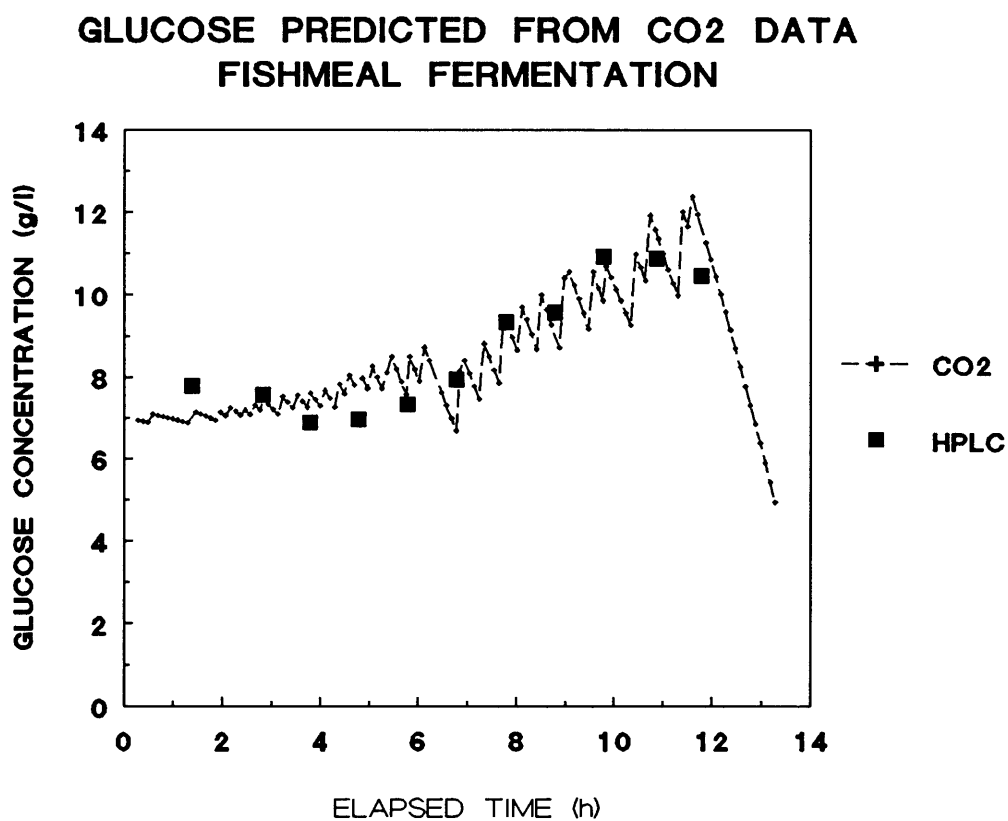


Figure 29: The prediction of glucose concentration in a fishmeal fermentation using the K_A value calculated from the same data.

3.3.3) Dissolved Oxygen Control Strategy

Bacillus subtilis is an aerobe and dissolved oxygen limitation may limit the growth of the cells and also interfere with protease production. Therefore, in all fermentations, it was attempted to maintain the dissolved oxygen above 20% saturation. This became progressively more difficult the higher the cell concentration. Typically, if the dissolved oxygen dropped below 20% the agitation rate was increased. Then once the limit of the agitation system had been reached (at about 700 rpm) the air flowrate was increased. Increases in air flowrate and agitation caused the additional problems of high gas holdup and excessive foaming so these strategies were often useful only for a short period during the fermentation.

To overcome these problems and to maintain the dissolved oxygen at high cell concentrations, oxygen enrichment of the inlet gas to the fermentor was used. Pure oxygen was mixed with air. The ratios of the air to oxygen were set so that the maximum concentration of oxygen in the inlet gas that could be reached was 50%. This was to avoid toxic high local oxygen concentrations that would develop should pure oxygen be used.

A proportional gain control scheme was used to control the oxygen/air ratio at a fixed gas flowrate as follows:

$$F_{O_2,i} = F_{O_2,i-1} + K_P \cdot (DO_{set} - DO_{actual}) \quad (10)$$

where

$F_{O_2,i}$: New flowrate of oxygen (l/min)

$F_{O_2,i-1}$: Flowrate of oxygen at the last scan (l/min)

K_P : Oxygen proportional control gain factor (l/min.%)

DO_{SET} : Dissolved oxygen setpoint (% of saturation)

DO_{ACTUAL} : Dissolved oxygen actually measured (% of saturation)
and

$$F_{AIR} = F_T - F_{O_2,i} \quad (11)$$

F_{AIR} : New flowrate of air (l/min)

F_T : Total flowrate required (l/min)

The oxygen and air flowrates were updated every 5 minutes and the maximum change in oxygen flowrate that could occur per scan was set at 0.25 l/min. The value of K_p used was 0.025 l/min-%.

Disadvantages of the Dissolved Oxygen Control Strategy

The control of dissolved oxygen based on gas mixing worked very well except when the computer crashed during a run. In this case the program would have to remember what the last oxygen flowrate was before the crash otherwise it would restart the oxygen flowrate at 0 l/min and it would take considerable time for the oxygen gas flow to reattain the desired level. If this time was too long then oxygen limitation could occur. In other words the scan time, gain factor and maximum permitted oxygen flowrate change required for start up after a crash were very different to the values needed during normal operation. Both sets of values need to be included in the strategy.

Excessive foaming sometimes increased the pressure in the vessel leading to higher dissolved oxygen readings and also led to fluctuation in the dissolved oxygen reading as the foam was controlled by antifoam addition. The gain and scan interval should be adjusted so that such variations do not lead to oscillations in the oxygen flowrate.

3.3.4) pH Control

The pH was controlled at 7 during all fermentations by the addition of NaOH. At the end of the fermentation the pH of the culture medium typically rose slightly above neutrality. This has been observed by other workers and is thought to occur during the transition of vegetative cells to sporulating cells during which time organic acids in the medium are oxidized by terminal respiratory reactions to high energy compounds. The production of these high energy compounds being a prerequisite to proper spore development and maturation (Bulla and Hoch, 1985).

3.3.5) Aeration Control

The gas flowrate was controlled to provide a 1.0-1.2 vvm (volume per volume per minute) to the fermentor regardless of the fermentation volume. The fermentation volume typically changed due to glucose, base and fishmeal additions and also due to sampling. The volumetric based flowrate (vvm) was kept constant by the on-line measurement of fermentation volume using a balance underneath the fermentor. This fermentation volume was used by Paragon to control the gas flowrate to the fermentor. As keeping the dissolved oxygen above its set point became difficult the vvm would be increased from 1.0 to 1.2.

3.3.6) Agitation Control

The agitation was controlled manually. Typically during a fermentation the agitation would start at about 500 rpm then as the dissolved oxygen decreased the agitation would be increased until 700 rpm. Agitating the viscous fishmeal fermentation above 700 rpm would

lead to the motor drive overheating.

3.3.7) Foam Control

The build up of foam was a considerable problem during some fermentations and the level of foam was controlled manually by the addition of polypropylene glycol (mw 2000, cat # 4787, Lot # 61350, Polysciences Inc., Warrington, PA 18976-2590).

3.3.8) Temperature Control

The temperature was controlled at 34°C during all fermentations using cooling water supplied to a jacket at the base of the fermentor.

3.4) Culture Handling and Media

3.4.1) Culture Storage

The fact that *Bacillus subtilis* is an endospore forming organism made storage convenient and easy. The culture can be stored as spores for considerable periods of time and regular transfers and subculturing avoided. Special medium is required to achieve good sporulation on a plate. Sporulation requires exogenous sources of calcium and manganese and is repressed by high levels of good carbon, nitrogen and phosphorus sources (Dean and Zeigler, 1989).

As recommended by the *Bacillus* Genetic Stock Center (Dean and Zeigler, 1989), Schaeffer sporulation medium, see section 3.4.2, was used to prepare sporulation plates. The culture, obtained from the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852), was reactivated from freeze dried form using ATCC guidelines and then plated on the sporulation plates. The sporulation plates were incubated at 30°C for 2 days then stored at room temperature for a further 2 days. After this the plates were sealed with masking tape, then sealed in plastic bags and stored at 4°C. The biggest problem with this storage method was desiccation of the agar medium, but cultures stored in this manner could last 1-2 years. Several subculturings were performed during the course of the study to ensure that the plates did not overly desiccate and that contamination was not occurring.

As a precaution against loosing the organism, spores from sporulation plates were suspended in sterile 10% (vol/vol) glycerol in water and stored at -70°C.

3.4.2) Media

Schaeffer Sporulation Medium (Schaeffer, 1965, Dean and Zeigler, 1989)

8 g/l	Nutrient broth
0.25 g/l	MgSO ₄ ·7H ₂ O
1 g/l	KCl
20 g/l	Bacto Agar

After autoclaving, add from separate sterile stocks

1 ml	FeSO ₄ ·7H ₂ O 10 ⁻³ M (filter-sterilized)
1 ml	MnCl ₂ ·4H ₂ O 10 ⁻² M
1 ml	CaCl ₂ ·2H ₂ O 1 M

Nutrient Agar Plates Used to Germinate Spores and Obtain Single Colonies

23 g/l	Nutrient agar
5 g/l	Bacto Agar

Test Tube Inoculum Medium

20 g/l	Glucose
18 g/l	Nutrient broth
1.3 g/l	K ₂ HPO ₄

Nutrient Broth Shake Flask Inoculum Medium

20 g/l	Glucose
18 g/l	Nutrient broth
1.3 g/l	K ₂ HPO ₄
0.5 g/l	MgSO ₄ ·7H ₂ O
0.5 ml/l	Polypropylene glycol antifoam

Fishmeal Shake Flask Inoculum Medium

20 g/l	Glucose
30 g/l	Raw fishmeal
1.3 g/l	K_2HPO_4
0.5 g/l	$MgSO_4 \cdot 7H_2O$
0.5 ml/l	Polypropylene glycol antifoam

Nutrient Broth Fermentation Medium

5 g/l	Glucose
25 g/l	Nutrient broth
2.3 g/l	K_2HPO_4
1.2 g/l	$MgSO_4 \cdot 7H_2O$
0.5 ml/l	Polypropylene glycol antifoam

Fishmeal Fermentation Medium

8.7 g/l	Glucose
40.4 g/l	Raw fishmeal
3.4 g/l	K_2HPO_4
1.8 g/l	$MgSO_4 \cdot 7H_2O$
0.5 ml/l	Polypropylene glycol antifoam

Feed Chemicals

600 g/l	Glucose feed
250 g/l	NaOH feed

Booster Solutions

226 g/l	K_2HPO_4
120 g/l	$MgSO_4 \cdot 7H_2O$

There were some variations to the media shown above used in some experiments. This related to the concentrations of cells that were obtained during the fermentation. For example, the fishmeal fermentation medium is designed to support 30 gdcw/l cells. If the cell concentration limit of the medium was approached during the fermentation then concentrated K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ "booster" solutions were added to ensure that the culture was not sulfate or phosphate limited. Twenty five ml of these "booster" solutions supported an additional 10 gdcw/l of cell growth in a 5 l fermentation volume. If less than 30 gdcw/l of cells was required then the concentrations of K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ in the initial medium were scaled down proportionately. It should be noted that the fishmeal also provided sulfate and phosphate as well as a variety of other trace nutrients.

Further variation in the medium from that shown above was used. Since the effect of fishmeal addition was being studied the initial concentration of fishmeal in the fermentation medium varied between experiments. Also, slightly different concentrations of glucose initially present in the medium and in the glucose feed were used as the glucose control strategy was developed. The media shown above are representative of the fermentations carried out and these media were actually used in particular experiments.

The fishmeal used in these media was obtained in 50 lb bags from Ventura Grain Inc. (148 Longmeadow Road, Tauton, MA 02780). The fishmeal, if suspended in water, had a dry weight of 70%. This meant that if a solid fishmeal concentration of 10 g/l was required in the fermentation broth, then 14.3 (10/0.7) g/l of fishmeal needed to be added. The concentrations of fishmeal used in the above media represent the actual amount of fishmeal, from the bag, that needed to be added to the medium, not the solid fishmeal

concentration initially in the medium. The fishmeal concentrations estimated during the fermentations are on a dry weight basis, all other fishmeal concentrations are based on the total amount of fishmeal added.

The media used to grow cultures of *Saccharomyces cerevisiae* and *Escherichia coli* can be found in O'Connor, 1989 and McMillan, 1990 respectively. The medium for the growth of *Saccharomyces cerevisiae* described in O'Connor, 1989 was scaled according to how much cell mass was required.

3.4.3) Inoculum Development and Fermentor Preparation

The inoculum was developed as follows. Spores from a spore plate were streaked onto a nutrient agar plate and incubated for 2 days at 30°C to give single colonies. Single colonies were then transferred to 10 ml of nutrient broth medium in a test tube. These test tubes were agitated for 12 hours on a reciprocating shaker at 30°C. After 12 hours the morphology of the cells was typically very long chains, although if the culture was left longer than 12 hours single cell morphology could be observed.

The 10 ml test tube culture was then transferred to a 500 ml baffled shake flask (catalog number 2543-04000, Bellco Glass Inc., 340 Edrudo Rd, Vineland, NJ 08360) culture. The shake flask was agitated on a reciprocating shaker at 30°C for 10-12 hours. This 500 ml shake flask culture was then used to inoculate the fermentor.

The fermentor was prepared for autoclaving containing fishmeal and K_2HPO_4 . The $MgSO_4 \cdot 7H_2O$ and glucose were autoclaved together but separate from the mixture in the fermentor. The $MgSO_4 \cdot 7H_2O$ and glucose solution was then added to the fermentor

after autoclaving.

The timing of inoculation was critical. If the inoculum was entering the stationary phase when it was added to the fermentor then a long lag phase could result. The ideal case would be to add an exponentially growing inoculum to the fermentor. Using *Bacillus subtilis* procured a unique advantage when it came to inoculation. Cell cultures of high growth rate were in a long chain like morphology whereas cultures of low growth rate were in a single cell morphology. This made identifying rapidly growing cultures easy. The cells were simply viewed under the microscope. Three separate inoculum cultures were prepared for each fermentation. When it came time to inoculate the most rapidly growing culture was selected to inoculate the fermentor.

Two types of fermentation using different types of medium were carried out in this study. Cells were grown without solid substrate present (using the nutrient broth fermentation medium), to identify metabolic parameters or to investigate the light scatter properties of known amounts of cells. Cells were also grown in the presence of solid substrate (using the fishmeal fermentation medium), to test the light scatter technology or to evaluate solid substrate utilization and product formation.

The various stages of the life cycle of *Bacillus subtilis* var *sakainensis* can be seen in figures 30, 31 and 32.

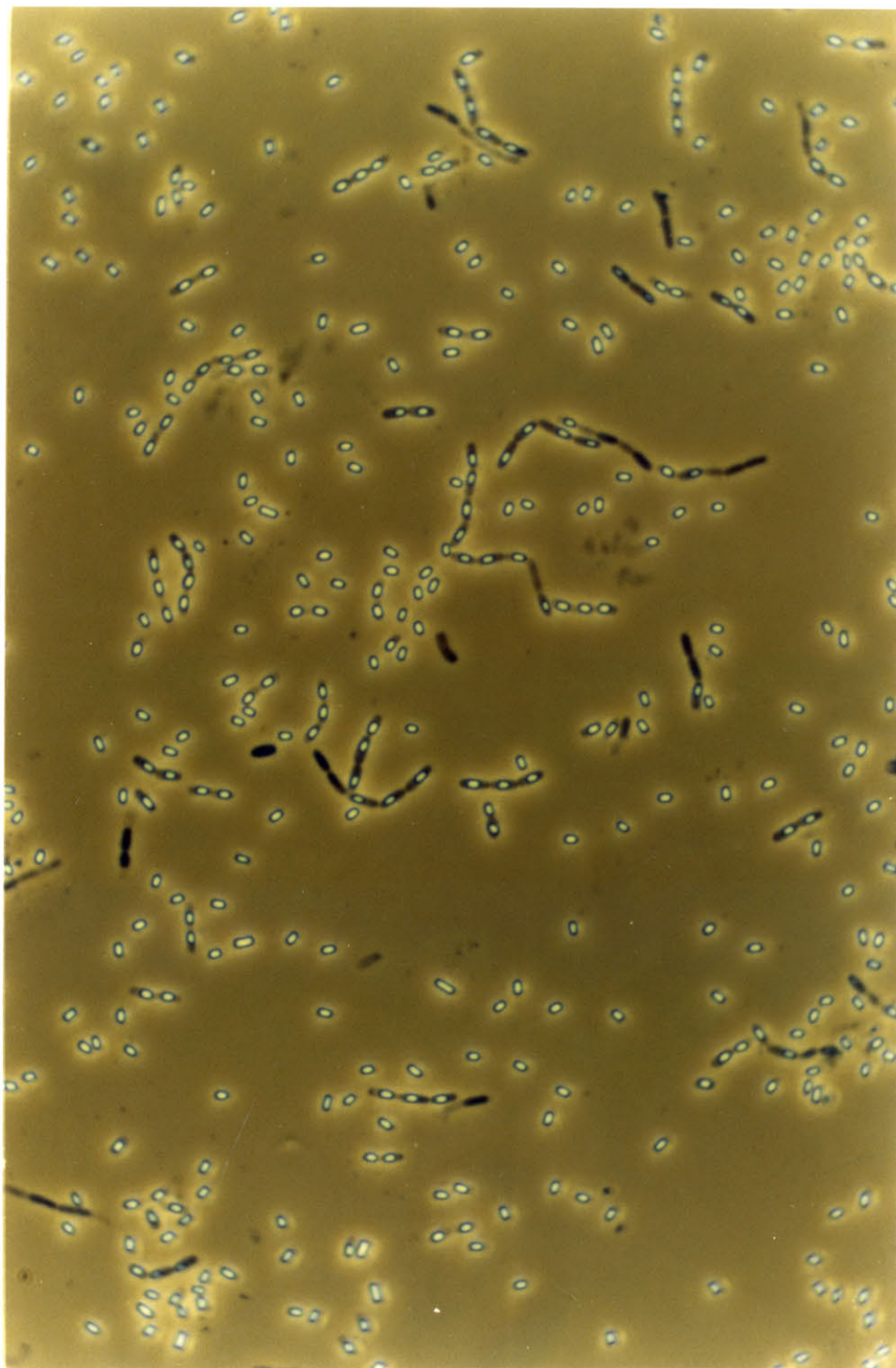


Figure 30: Spores of *Bacillus subtilis* var *sakainensis* ATCC 21394 taken from a sporulation agar plate. Taken under oil at 1000x magnification.

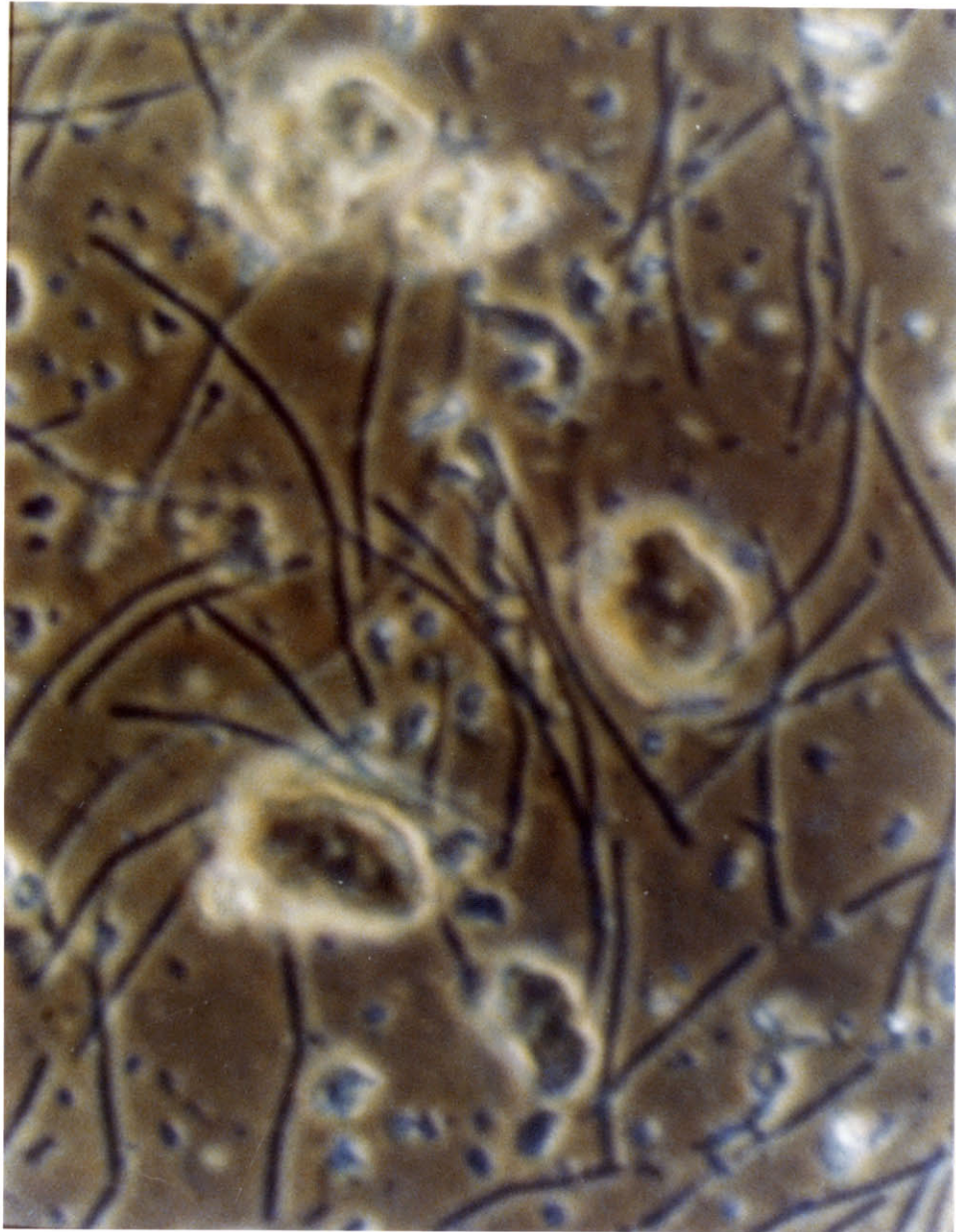


Figure 31: Chains of *Bacillus subtilis* var *sakainensis* ATCC 21394 growing on fishmeal near the beginning of the fermentation. Taken under oil at 1000x magnification.

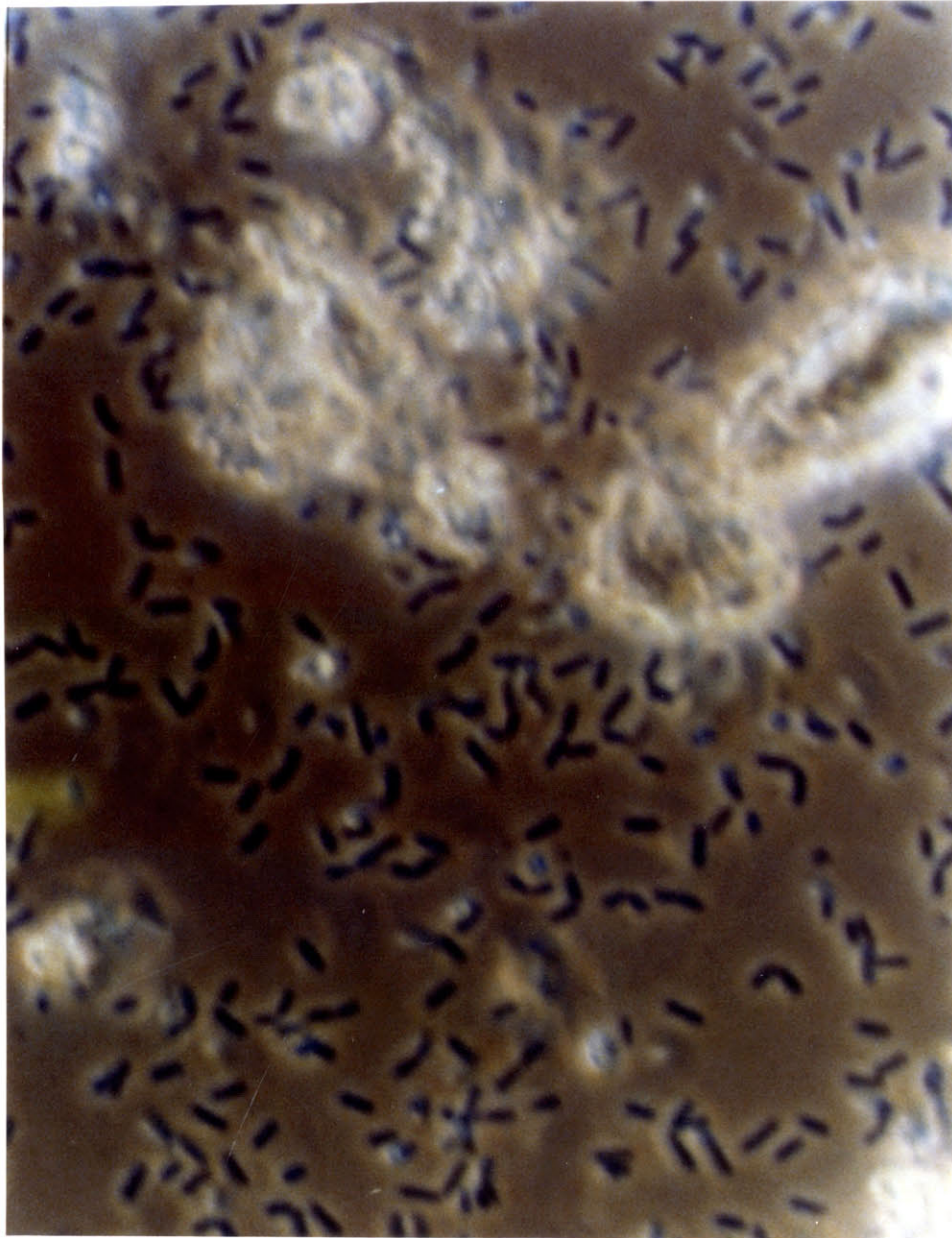


Figure 32: Single cells of *Bacillus subtilis* var *sakainensis* ATCC 21394 growing on fishmeal near the end of the fermentation. Taken under oil at 1000x magnification.

4) RESULTS AND DISCUSSION

4.1) The Light Scatter Spectra of Cells and Solids

The light scatter spectrum is the light scatter readings as a function of wavelength between 330 and 680 nm and is based on earlier observations by Junker et al., 1988. The light scatter spectrum of the individual fermentation broth components, (at approximately the same concentration) was investigated. The light scatter spectra of three different cell types, *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Escherichia coli* can be seen in figure 33. All the cell spectra show three characteristic peaks at 380, 540 and 610 nm. The sources of these characteristic peaks, whether related to the cellular constituents or machine variables, are not known. Interestingly the spectrum of *Bacillus subtilis* exhibits an additional peak at or below 330 nm. The magnitude of the scatter from *Escherichia coli* and *Bacillus subtilis* is greater than that from *Saccharomyces cerevisiae*. A probable cause of this is that the size of the yeast is greater than that of the bacteria.

The light scatter spectra of different fermentation substrates can be seen in figure 34. Soybean meal exhibits the highest light scatter readings and cottonseed meal the lowest. The spectra of the fermentation substrates exhibit peaks at 540 nm and 610 nm, the same as seen in the cell spectra. However, the peaks at 330 nm and 380 nm seen in the cell spectra, do not appear in the substrate spectra.

No rule of thumb could be determined for the a priori prediction of the light scatter spectrum of a given substrate. The light scatter spectra did not correlate with any particular observable property. Attempts at relating light scatter properties with characteristics observable in SEM photographs were not successful.

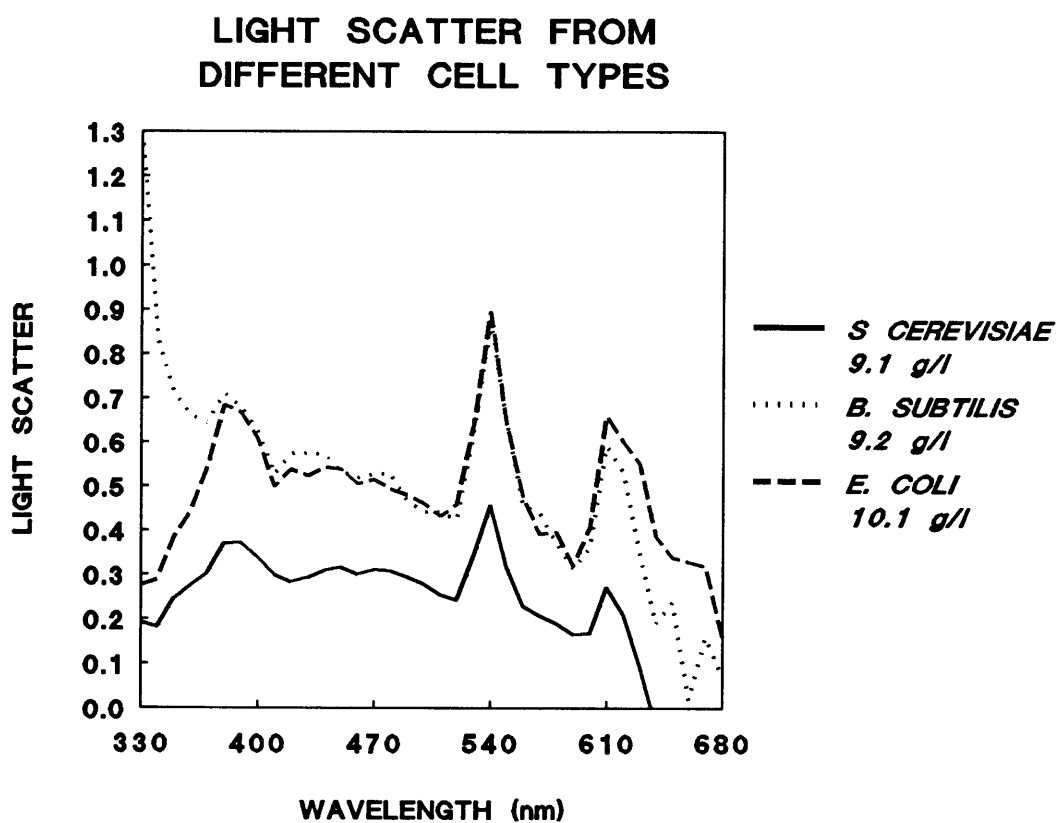


Figure 33: The light scatter spectra of *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Escherichia coli*.

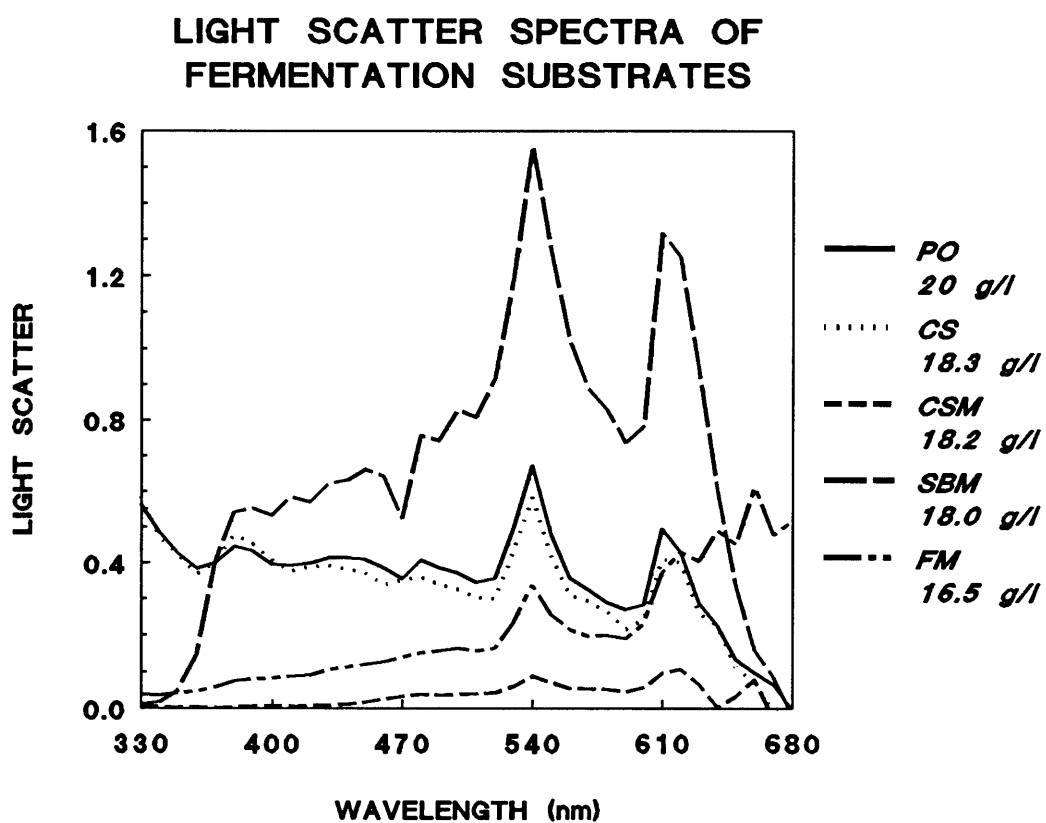


Figure 34: The light scatter spectra of fermentation substrates. Abbreviations: PO (peanut oil), CS (corn starch), CSM (cottonseed meal), SBM (soybean meal), FM (fishmeal).

4.2) The effect of Concentration on the Light Scatter Spectrum

The effect of cell concentration on the light scatter spectrum of *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Escherichia coli* can be seen in figures 35, 36 and 37 respectively. The increase in light scatter as a function of wavelength is nearly linear at most wavelengths. In order to reduce errors introduced by random fluctuations in a single light scatter reading, the area under a narrow (10 nm) interval of the light scatter spectrum was correlated with cell concentration. This area measurement is called the integrated light scatter reading or ILS.

The response of the integrated light scatter reading to cell concentration between 470-480 nm can be seen in figure 38. The curves of the two bacterium are very similar but different to the yeast. The relationship between integrated light scatter and cell concentration form the basis of cell concentration estimation. The figures 35, 36 and 37 are used as calibration data for the technique. A useable relationship between integrated light scatter and cell concentration was observed up to 50 g/l for *Bacillus subtilis*, see figure 39.

The effect of concentration of fishmeal and cottonseed meal on the light scatter spectrum can be seen in figures 40 and 41. The light scatter spectra of these solid substrates are much less sensitive to concentration than the light scatter spectra of cells. For equivalent concentrations the light scatter reading from the cells is much greater than that from the solid substrates.

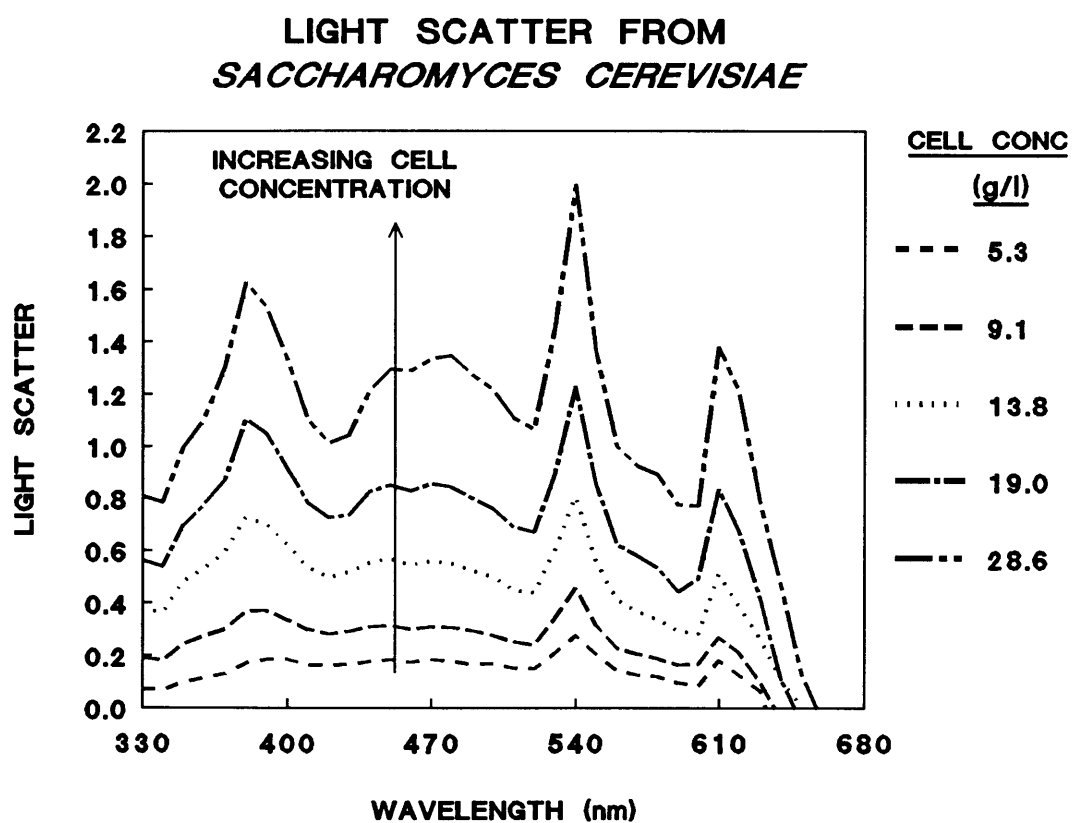


Figure 35: The effect of cell concentration on the light scatter spectrum of *Saccharomyces cerevisiae*.

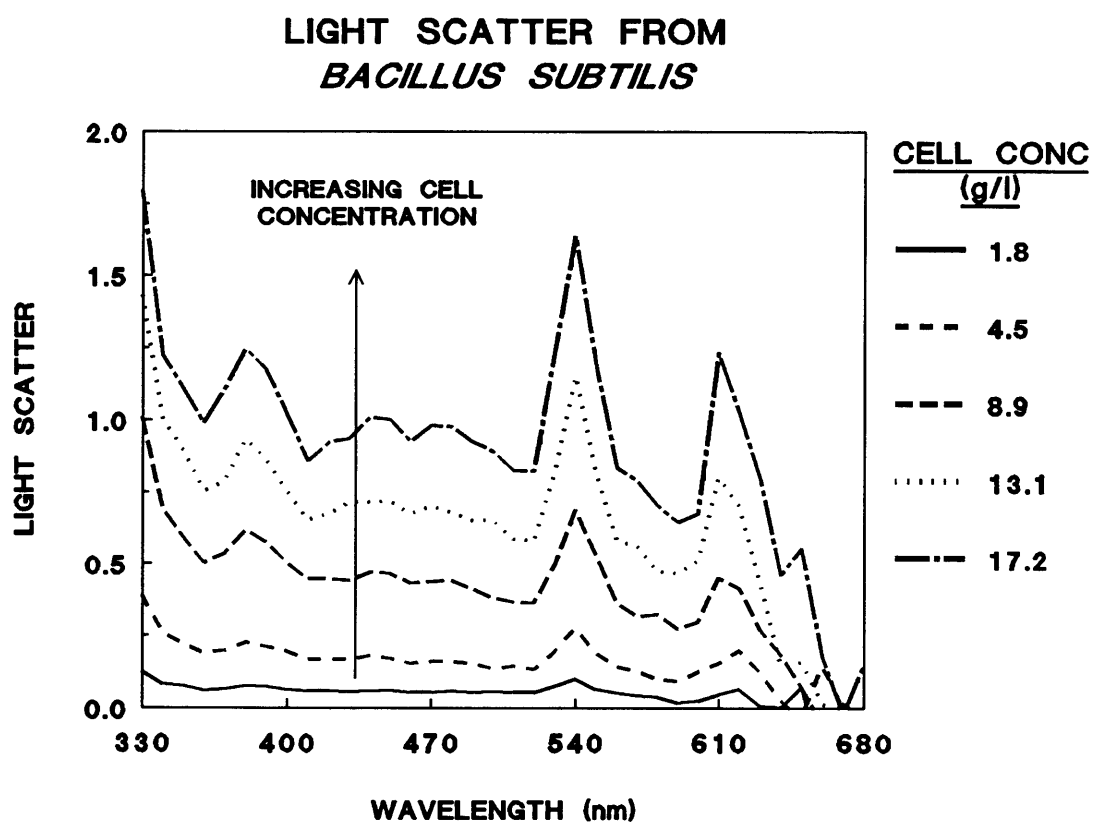


Figure 36: The effect of cell concentration on the light scatter spectrum of *Bacillus subtilis*.

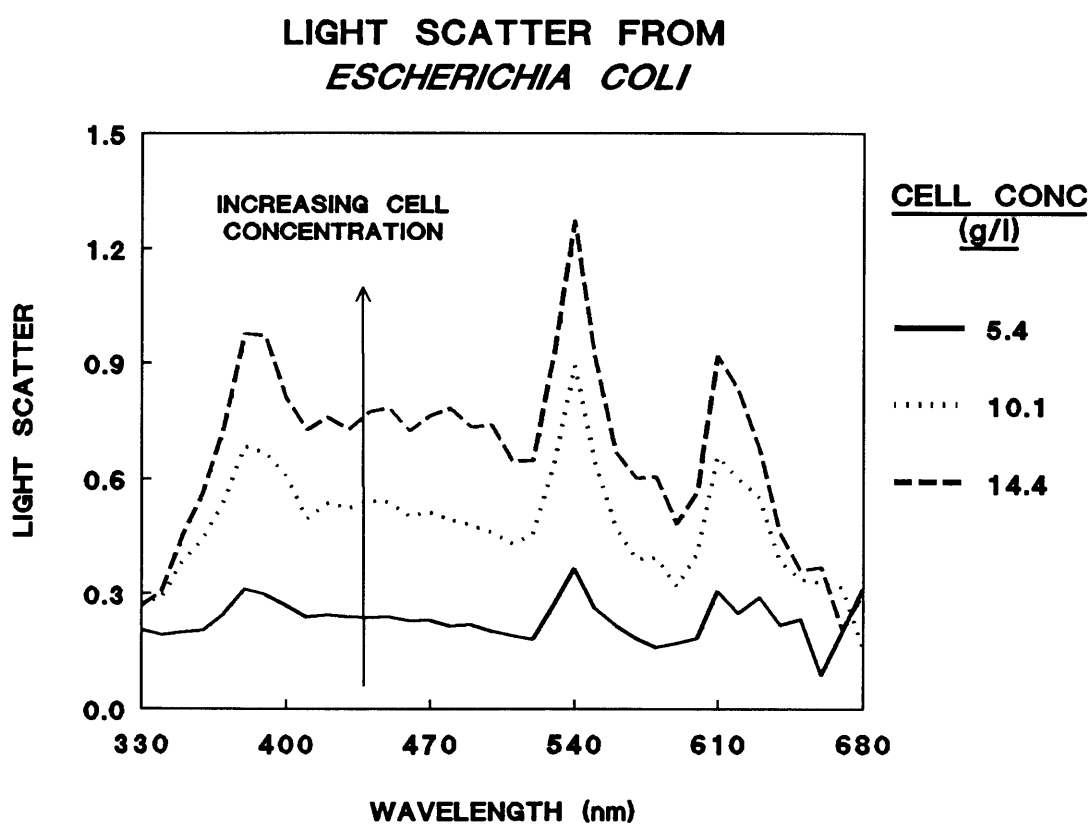


Figure 37: The effect of cell concentration on the light scatter spectrum of *Escherichia coli*.

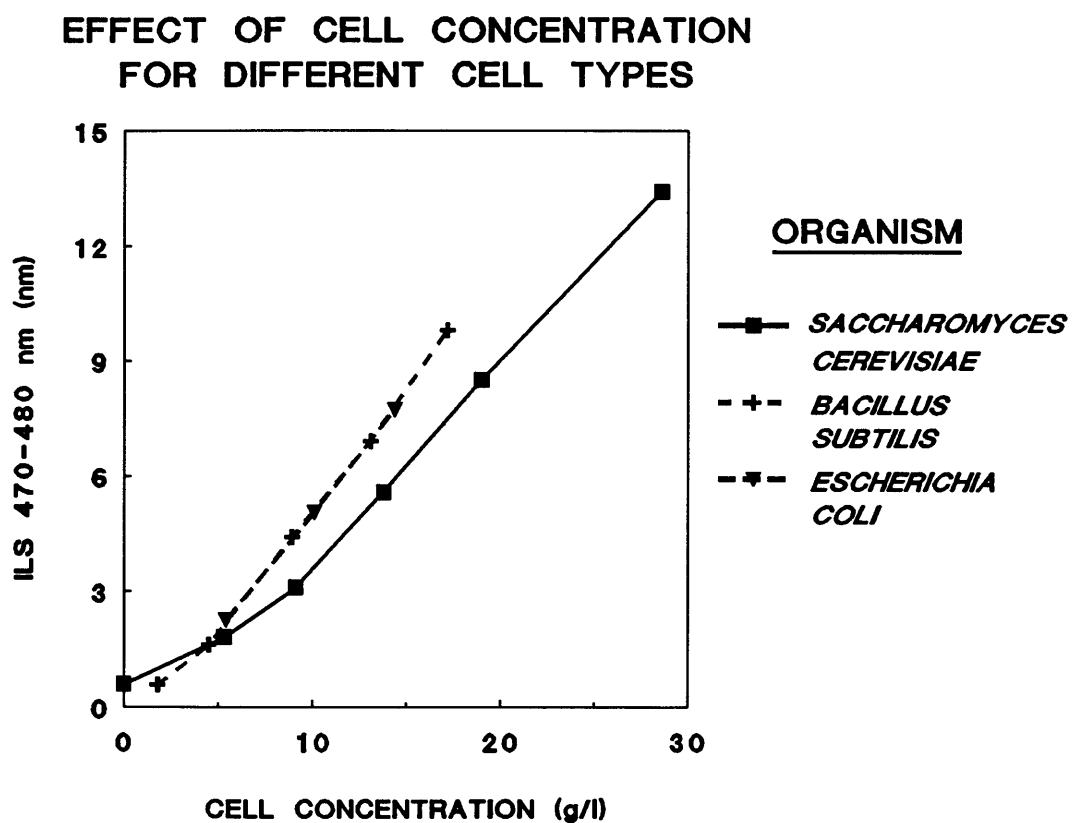


Figure 38: The response of integrated light scatter (470-480 nm) to cell concentration.

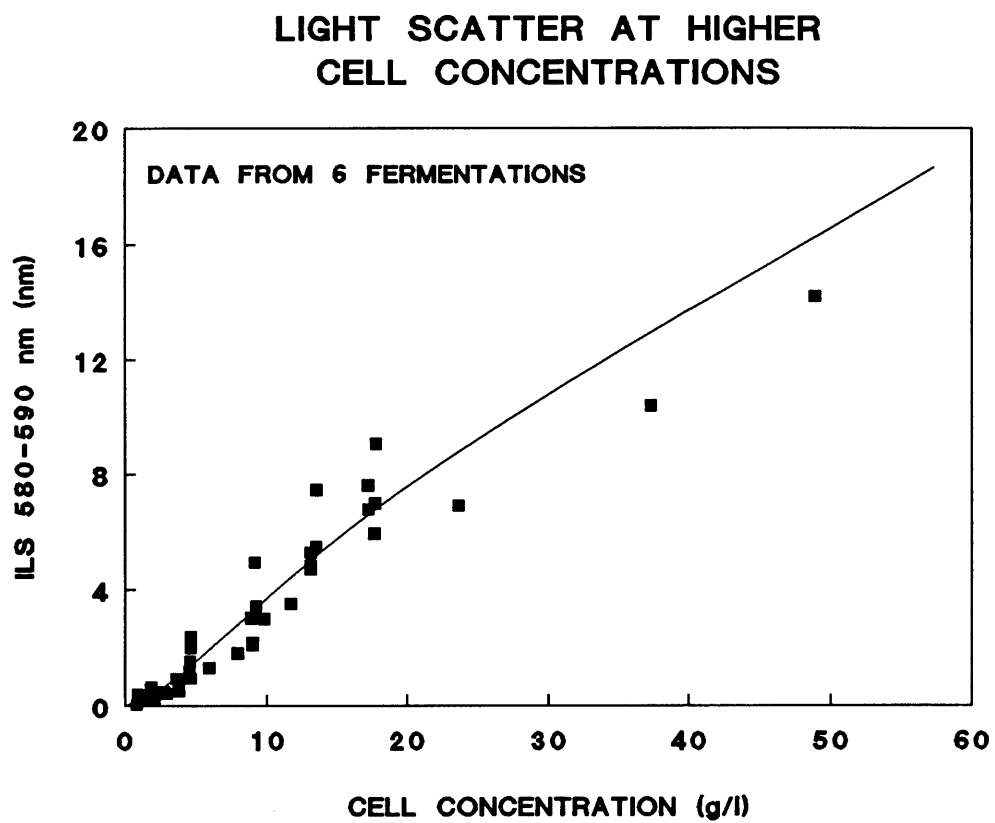


Figure 39: The effect of cell concentration on the integrated light scatter (580-590 nm) of *Bacillus subtilis* up to 50 g/l.

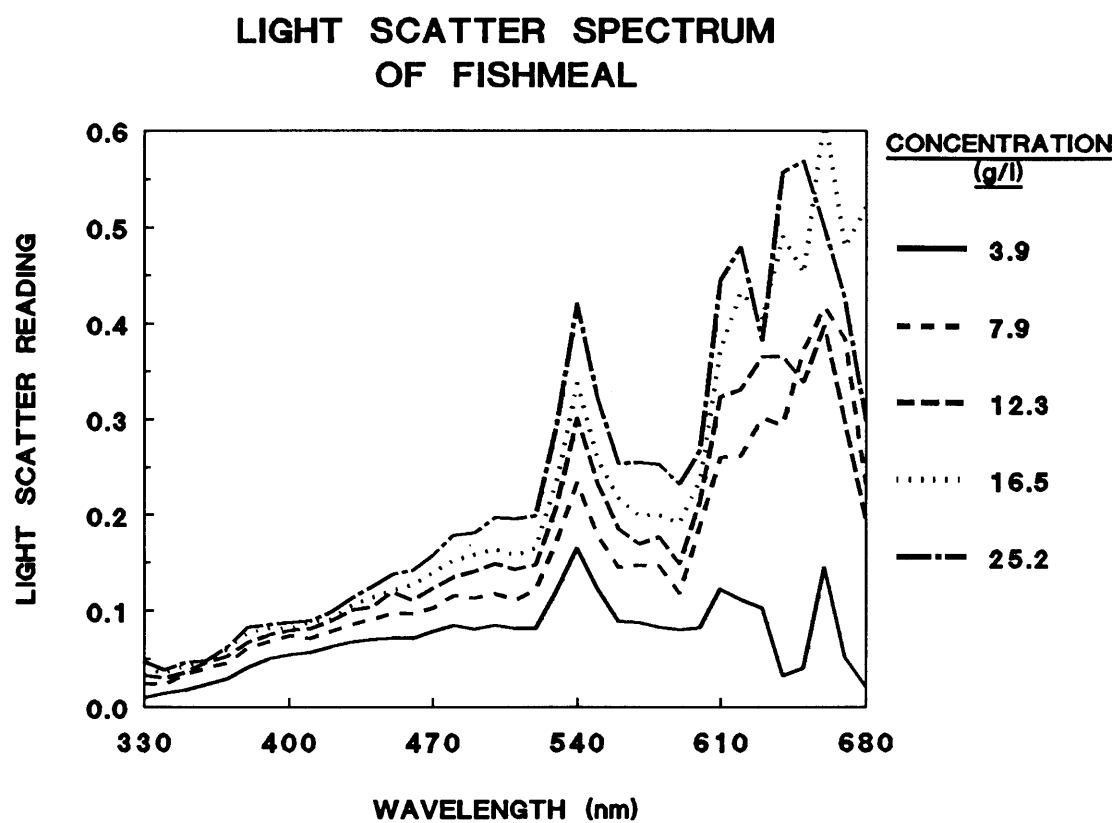


Figure 40: The effect of concentration on the light scatter spectrum of fishmeal.

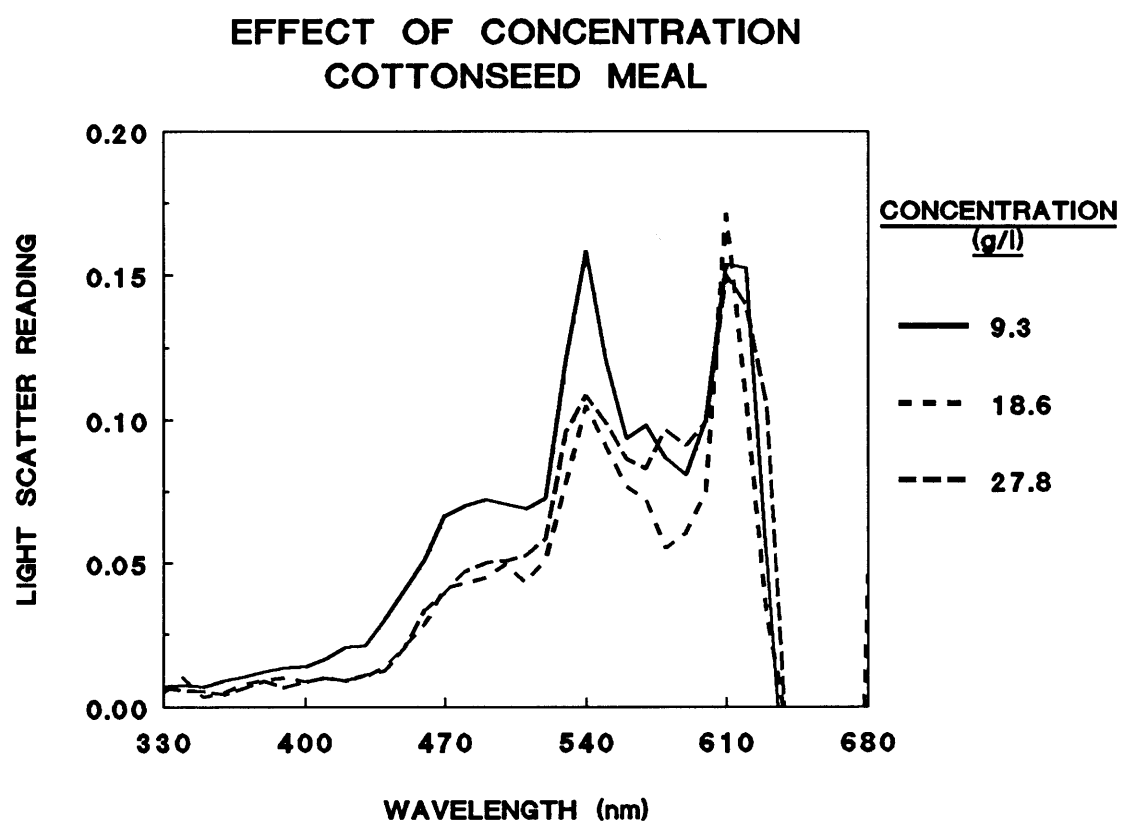


Figure 41: The effect of concentration on the light scatter spectrum of cottonseed meal.

4.3) The effect of Probe Geometry

The probe geometry used to measure light scatter greatly affects the results. Two different types of probe geometry were investigated. The front face probe, in which the scattered light was measured at 180° to the incoming light, and a right angle probe, in which the scattered light was measured at 90° to the incoming light. The two probe geometries can be seen in figure 42.

The front face probe was the most successful for our application because it had a near linear response to cell concentration even at high cell concentrations (>30 g/l). This meant that the probe could be used to measure industrially significant cell concentrations without dilution. The right angle probe in contrast gave a linear response to cell concentration only up to 4 g/l with *Bacillus subtilis*. Above 4 g/l the signal actually decreased with increasing cell concentration, see figure 43. This meant that the probe may have potential in situations where the cell concentration was very small. However, further investigations, see section 4.4, showed that invariant regions were not observed in spectra generated using a right angle probe.

All spectra shown in this thesis were obtained using a front face probe unless specifically indicated otherwise.

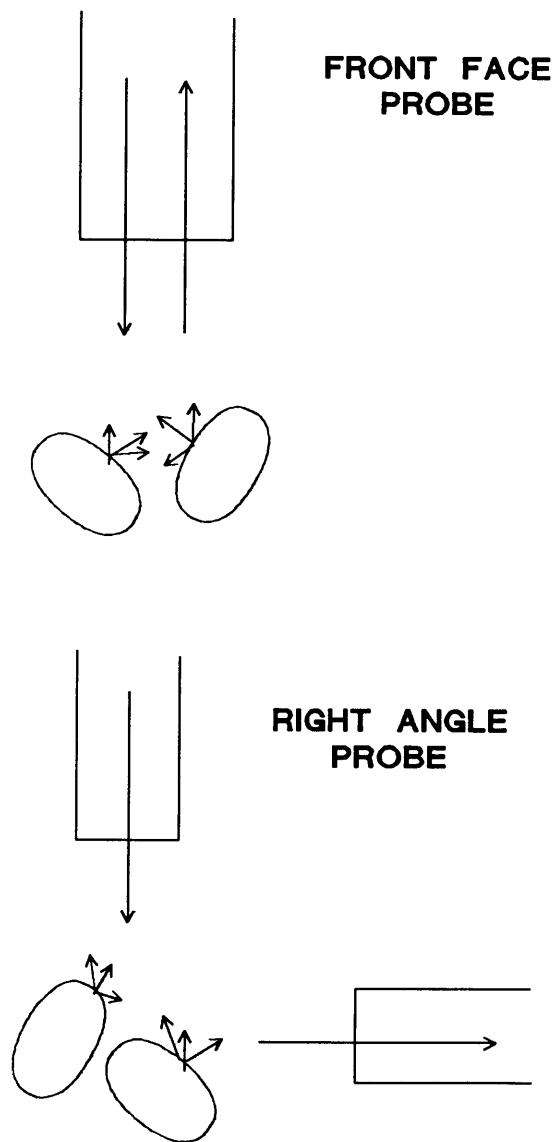


Figure 42: Front face and right angle probe geometries.

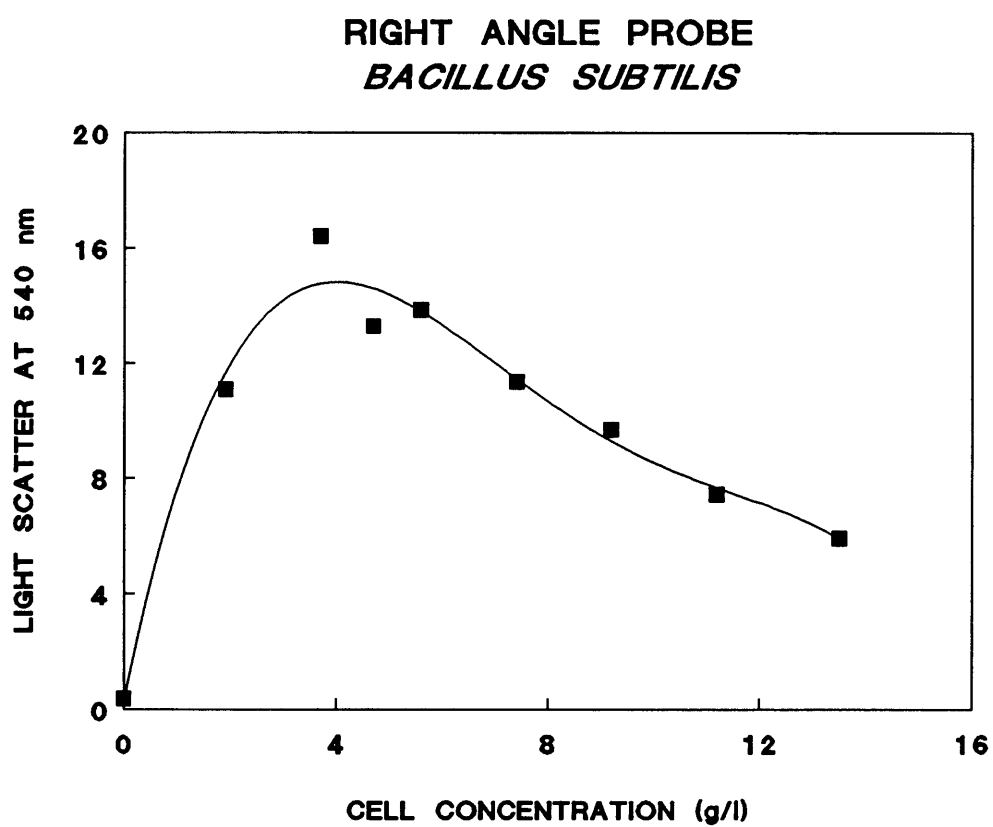


Figure 43: The light scatter at 540 nm as a function of *Bacillus subtilis* concentration obtained using a right angle probe.

4.4) The Light Scatter Spectrum of Mixtures of Cells and Solids

The light scatter spectra of cell and solid mixtures can be seen in figures 44 to 56. The light scatter spectra of cell and solid mixtures exhibit "invariant regions". These invariant regions can be seen in plots of the light scatter spectrum at constant cell concentration but varying solid concentration. Invariant regions are regions of the light scatter spectrum in which the light scatter reading is a function of the cell concentration but is independent of the solid concentration.

The existence of invariant regions can be explained as follows. Both cell and solid particles scatter and absorb light. At high wavelengths, towards 700 nm, solid substrate particles scatter more light than they absorb. Hence, adding solids to a suspension of cells, increases the light scatter. At low wavelengths, towards 330 nm, solid substrate particles absorb more light than they scatter. Hence, adding solid substrate to a suspension of cells, decreases the light scatter - the solid substrate has absorbed some of the light scattered by the cells.

At some intermediate wavelength the solid substrate must scatter as much light as it absorbs. Thus the light scatter at this wavelength is independent of the solid substrate concentration but is a function of the cell concentration. This invariant region provides a means of using light scatter to estimate cell concentration during solid substrate fermentations.

Invariant regions were observed for a variety of cell/solid substrate systems and at a number of different cell concentrations. The invariant region does not stay in one fixed location but its location is a function of the cell concentration. This can be seen in figures

44 to 55. Invariant regions can be observed in the light scatter spectrum of mixtures of *Bacillus subtilis* and fishmeal (figures 44 to 46), *Bacillus subtilis* and soybean meal (figures 47 to 49), *Saccharomyces cerevisiae* and fishmeal (figures 50 to 52) and *Escherichia coli* and fishmeal (figures 53 to 55). The solid concentrations shown in the experimental mixtures are on a moisture free basis. These spectra show that invariant regions are found with a variety of cell types and a variety of solid substrates, and that it is not a phenomenon isolated to a particular cell/solid combination. Invariant regions are also observed when more than one solid substrate is present in the medium, see figure 56.

Invariant region location as a function of cell concentration can be seen in figures 57 to 60. A summary of this data showing the center of the invariant region as a function of cell concentration can be seen in figure 61. The slope of the invariant region versus cell concentration graph, is linear between 4-20 g/l, see figure 57. In all systems studied this slope was approximately 10 nm.l/g, see table 3. The reason for this constant slope amongst different cell/solid combinations is not known.

Table 3: The linear slope between 4-20 g/l cells of the location of the invariant region as a function of cell concentration.

<u>Cell Type</u>	<u>Solid Type</u>	<u>Slope</u> (nm.l/g)
<i>Saccharomyces cerevisiae</i>	Fishmeal	9.6
<i>Escherichia coli</i>	Fishmeal	11.3
<i>Bacillus subtilis</i>	Fishmeal	10.1
<i>Bacillus subtilis</i>	Soybean meal	10.1

The following technique was used to determine the location of the invariant region

in the light scatter spectrum. The invariant region can be observed when the cell concentration is kept fixed and the solid concentration is changed. Four spectra of varying solid concentration (usually including a spectrum with no solid present) is sufficient to confirm the location of an invariant region. Once these four spectra have been recorded any one of the following three methods can be used to determine the location of the invariant region.

A) Percentage Criteria Method

At each wavelength, the four light scatter readings are averaged and the percentage deviation of each light scatter reading from this average are determined. The absolute values of these percentage deviations are again averaged. If the average absolute deviation from the mean is less than 10%, then the wavelength is considered to fall within the invariant region. Although 10% deviation is used as a standard criterion for determining whether a particular wavelength falls within the invariant region, this selection is somewhat arbitrary and other deviations can be used. This method tends to predict very narrow (10 nm) invariant regions at low cell concentrations and very wide invariant regions (60 nm) at high cell concentrations.

B) Crossover Criteria Method

In this method, at each wavelength, the light scatter reading of the cell only spectrum is compared to the three light scatter readings obtained for the cell/solid mixtures. If the cells only reading is above or below the other three readings, then the wavelength is not

within the invariant region. The wavelength of the cell only reading is deemed to be within the invariant region, if it is equal to or in between the other three readings. In other words, the light scatter reading at a particular wavelength for the cell only suspension must be located between the upper and lower light scatter readings in order for it to be within the invariant region.

C) Optical Criteria Method

The four spectra described above are plotted on one graph. Since the invariant region only occupies a small amount of the entire light scatter spectrum, the graph is enlarged around the invariant region, to increase accuracy. The location of the invariant region is then determined visually as being the point of intersection of all the light scatter spectra. The results with this method will vary slightly with observer but were observed to be within the invariant regions predicted by the percentage criteria method and the crossover criteria method.

For estimating the cell concentration of *Bacillus subtilis* growing on fishmeal during fermentation, the crossover criteria method was used to determine the location of the invariant region. For locating the invariant region in other systems either the crossover criteria method or the percentage criteria method was used. The three methods above are not an exhaustive list of means of locating the invariant region.

Invariant regions were not observed in all cell/solid mixtures. For example, *Bacillus subtilis*/corn starch and *Bacillus subtilis*/cottonseed meal, do not exhibit invariant regions

between 330-700 nm. This does not mean that invariant regions do not form in these systems but may mean that the invariant regions are outside the 330-700 nm observation window. Equipment limitations meant that the light scatter could not be measured above 700 nm or below 330 nm. The potential location of the invariant region, either above 700 nm or below 330 nm is easily estimated. If the cell only curve is on top of the cell/solid mixture spectra then, the invariant region is most probably above 700 nm (as is the case with *Bacillus subtilis*/cottonseed meal, see figure 62). If the cell only curve is below the cell/solid mixture spectra, then the invariant region is most probably below 330 nm (as is the case with *Bacillus subtilis*/corn starch, see figure 63).

An experiment was conducted to see if invariant regions were observed at high solid concentrations. Invariant regions were observed in mixtures of *Bacillus subtilis* and fishmeal, up to 126 g/l of fishmeal, see figure 64.

Invariant regions were not observed when using the right angle probe. This was true even in systems which exhibited invariant regions when using the front face probe, see figure 65. This precluded the use of the right angle probe to estimate cell concentration in the presence of solid substrate.

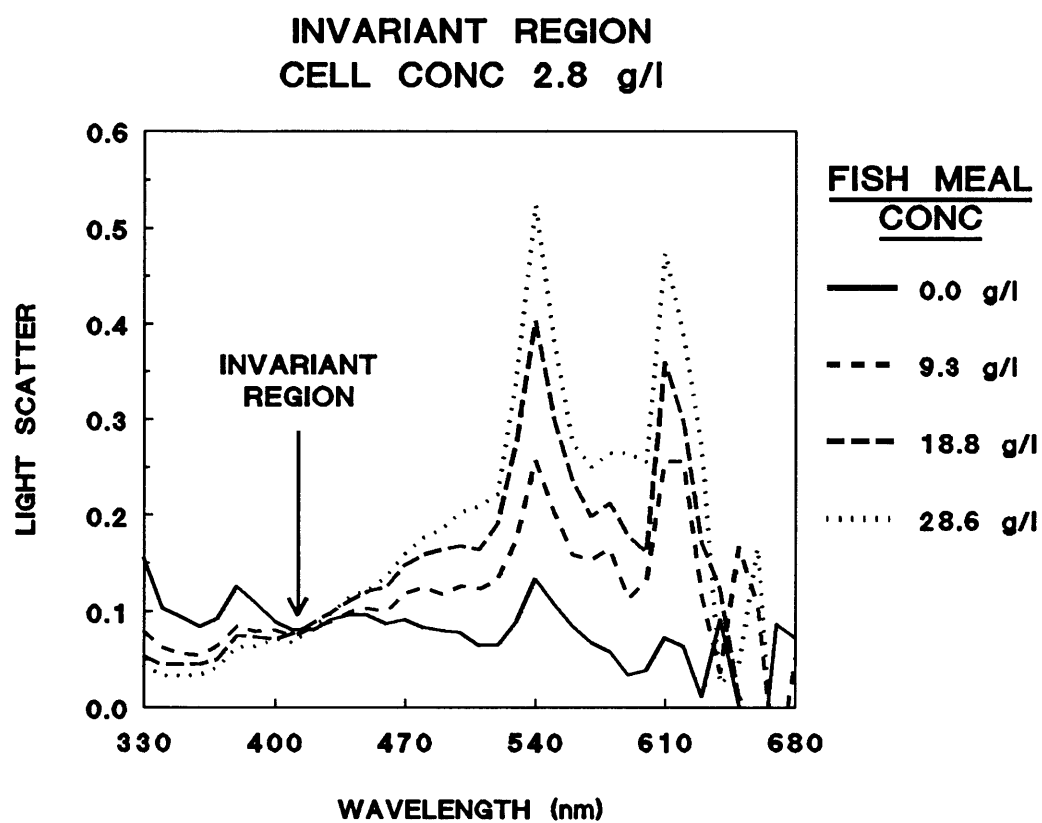


Figure 44: An invariant region in the light scatter spectra of mixtures of *Bacillus subtilis* and fishmeal at a cell concentration of 2.8 g/l.

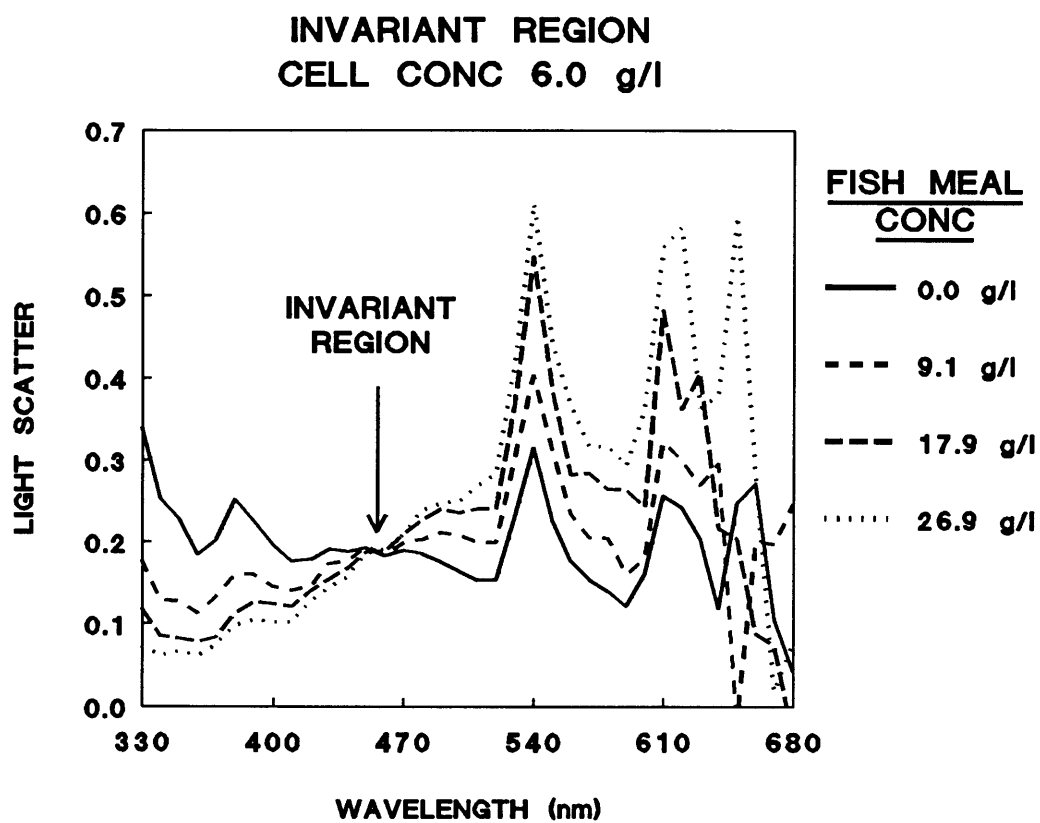


Figure 45: An invariant region in the light scatter spectra of mixtures of *Bacillus subtilis* and fishmeal at a cell concentration of 6.0 g/l.

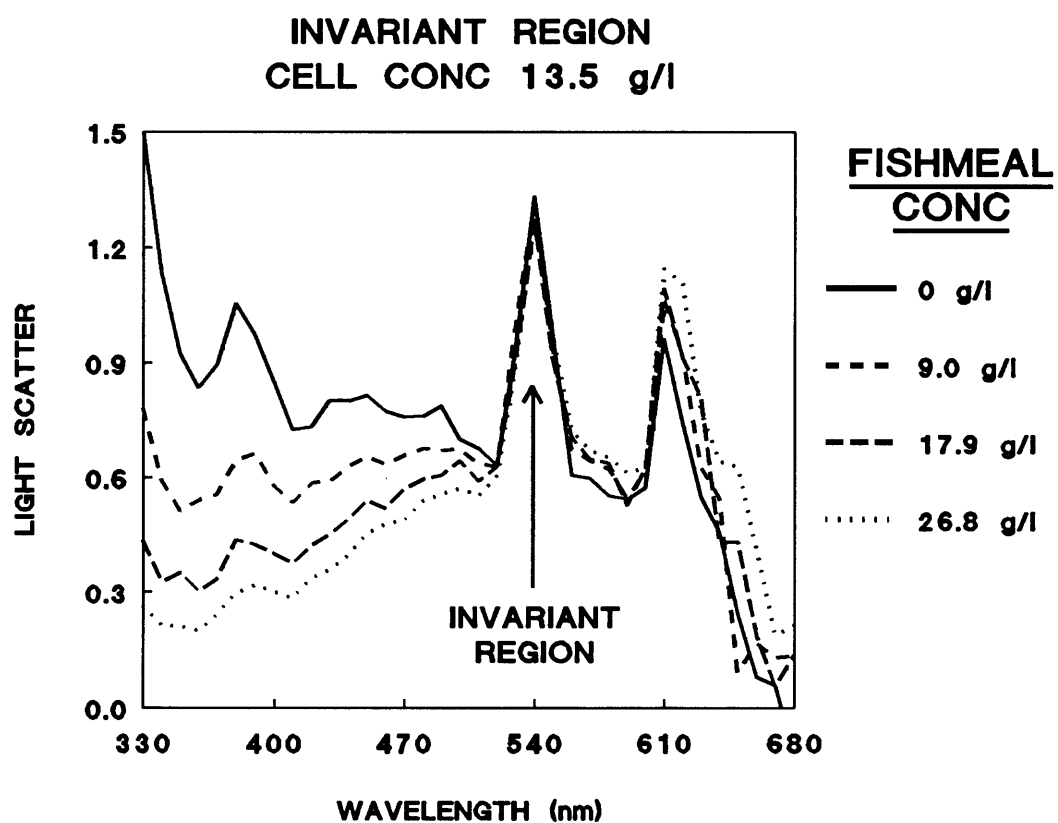


Figure 46: An invariant region in the light scatter spectra of mixtures of *Bacillus subtilis* and fishmeal at a cell concentration of 13.5 g/l.

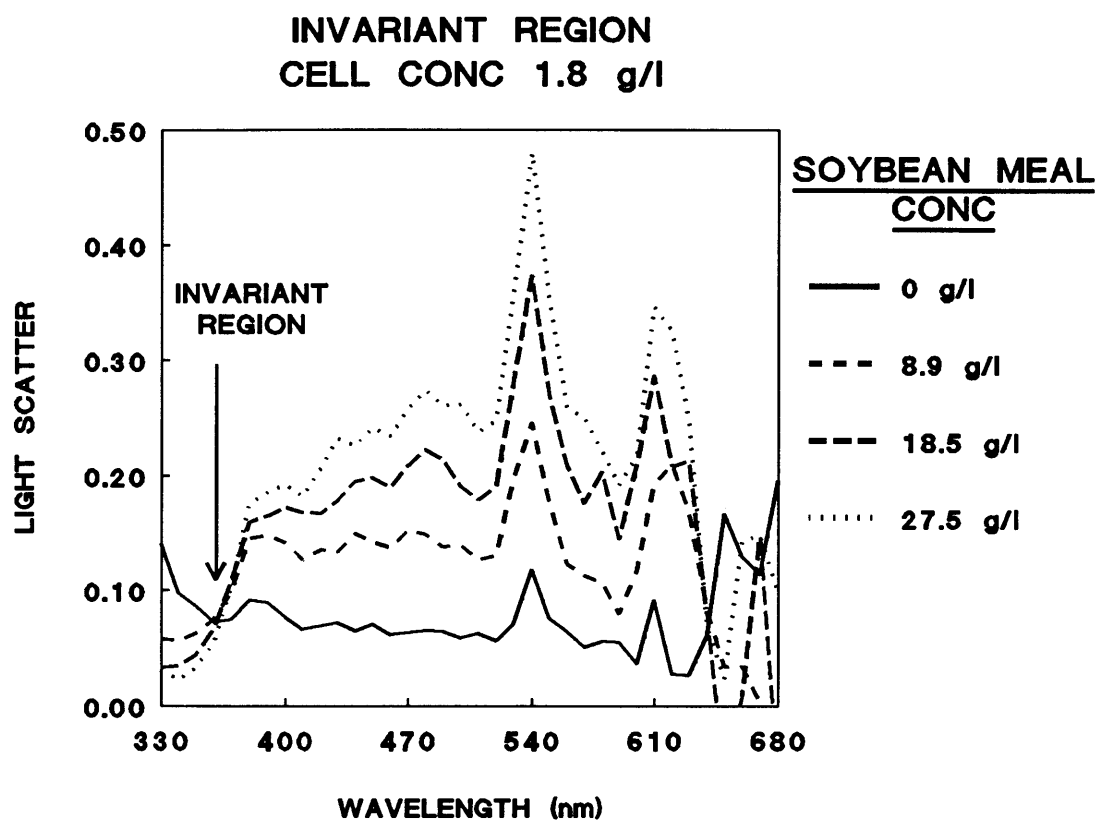


Figure 47: An invariant region in the light scatter spectra of mixtures of *Bacillus subtilis* and soybean meal (180-300 microns) at a cell concentration of 1.8 g/l.

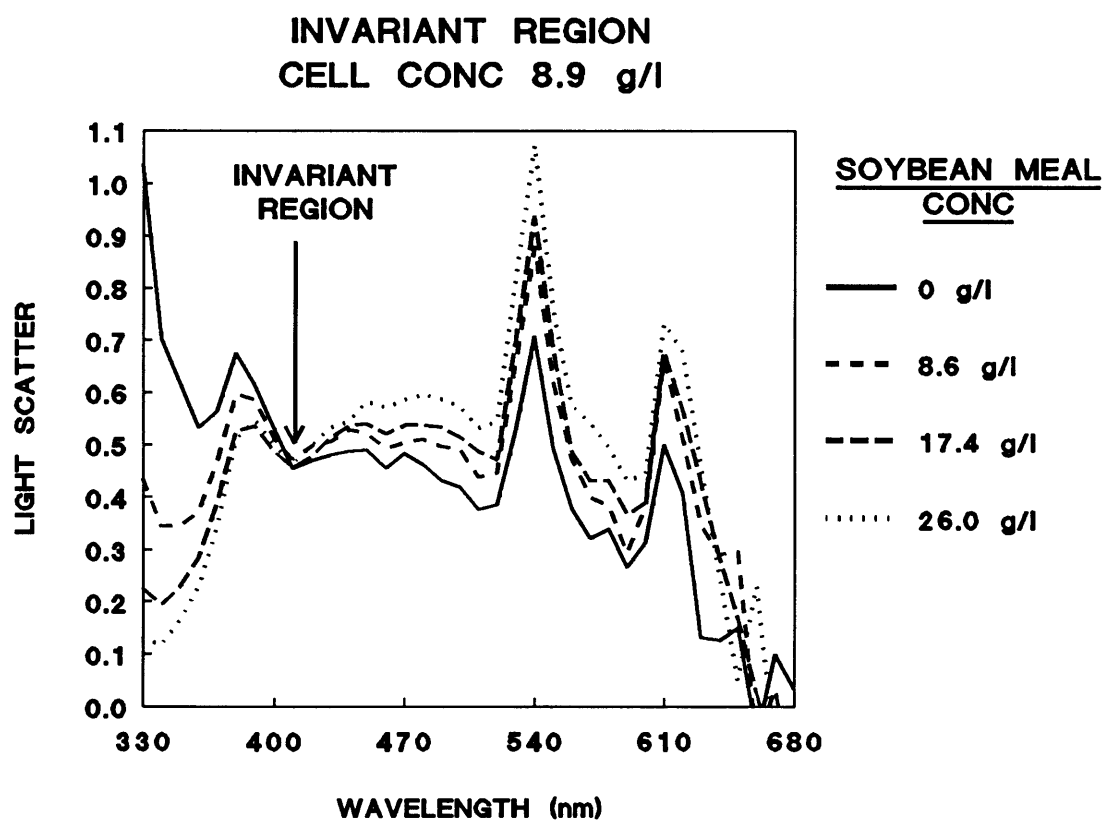


Figure 48: An invariant region in the light scatter spectra of mixtures of *Bacillus subtilis* and soybean meal (180-300 microns) at a cell concentration of 8.9 g/l.

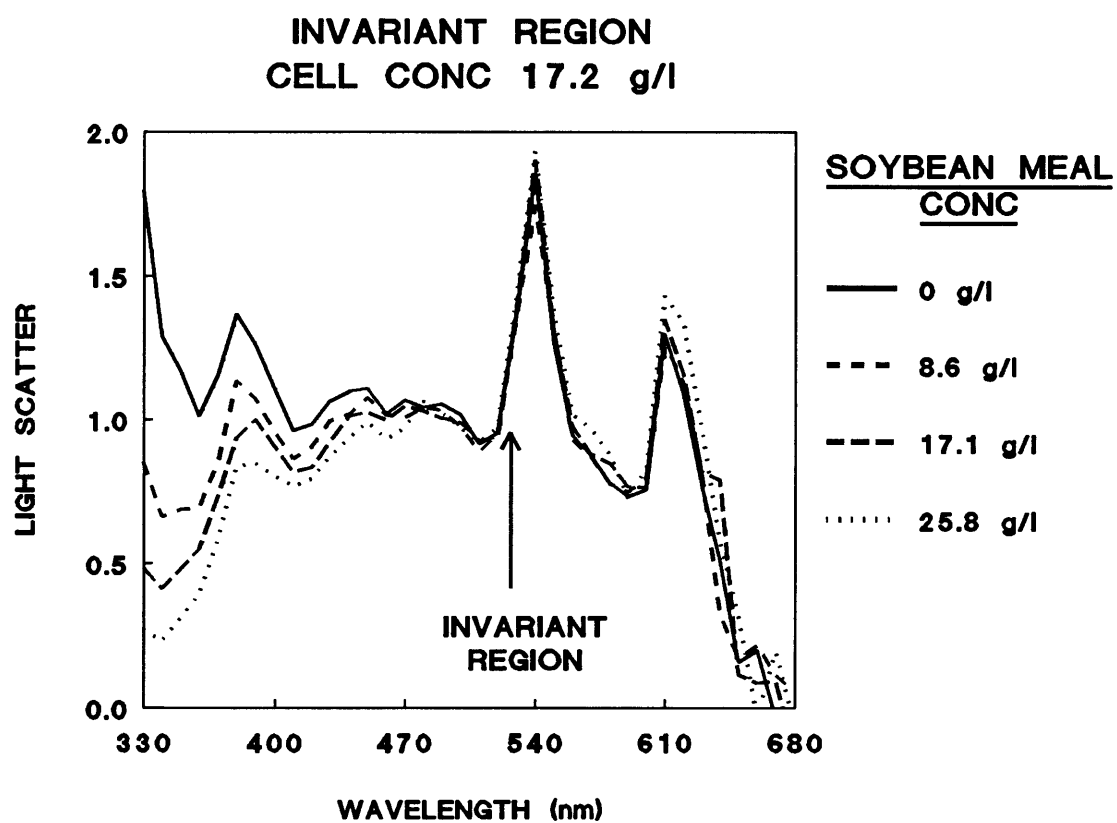


Figure 49: An invariant region in the light scatter spectra of mixtures of *Bacillus subtilis* and soybean meal (180-300 microns) at a cell concentration of 17.2 g/l.

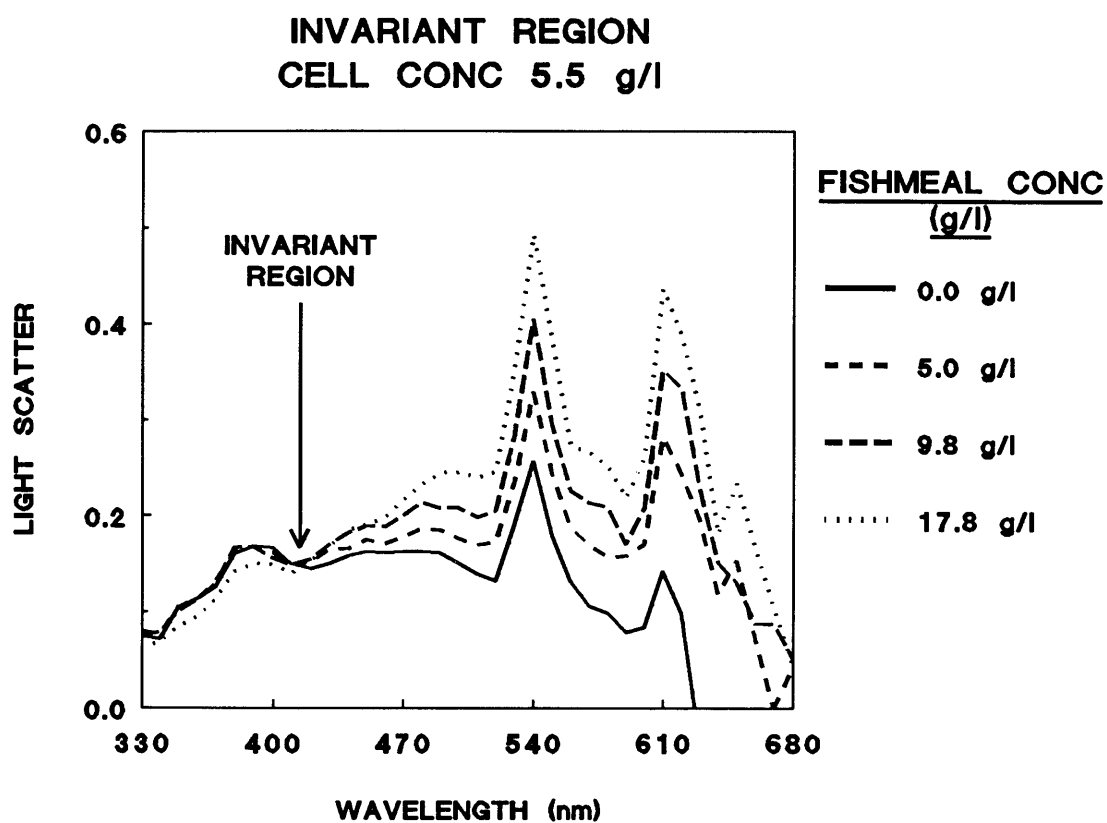


Figure 50: An invariant region in the light scatter spectra of mixtures of *Saccharomyces cerevisiae* and fishmeal at a cell concentration of 5.5 g/l.

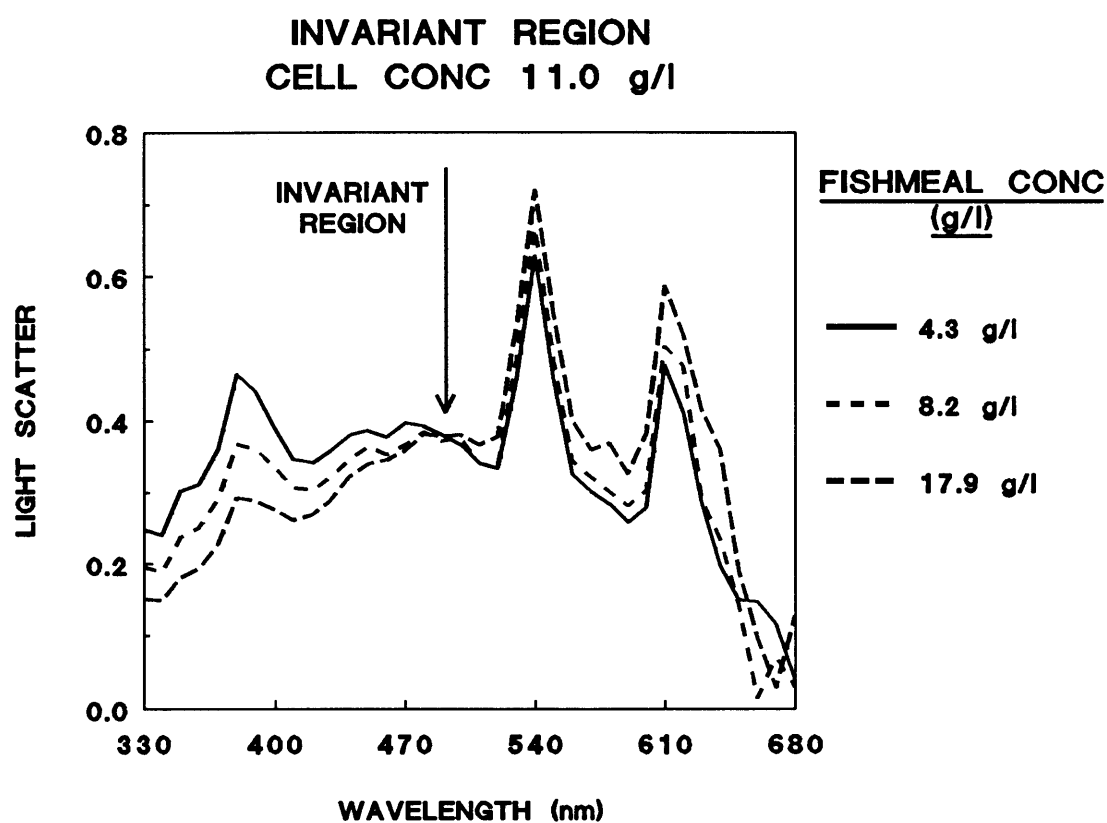


Figure 51: An invariant region in the light scatter spectra of mixtures of *Saccharomyces cerevisiae* and fishmeal at a cell concentration of 11.0 g/l.

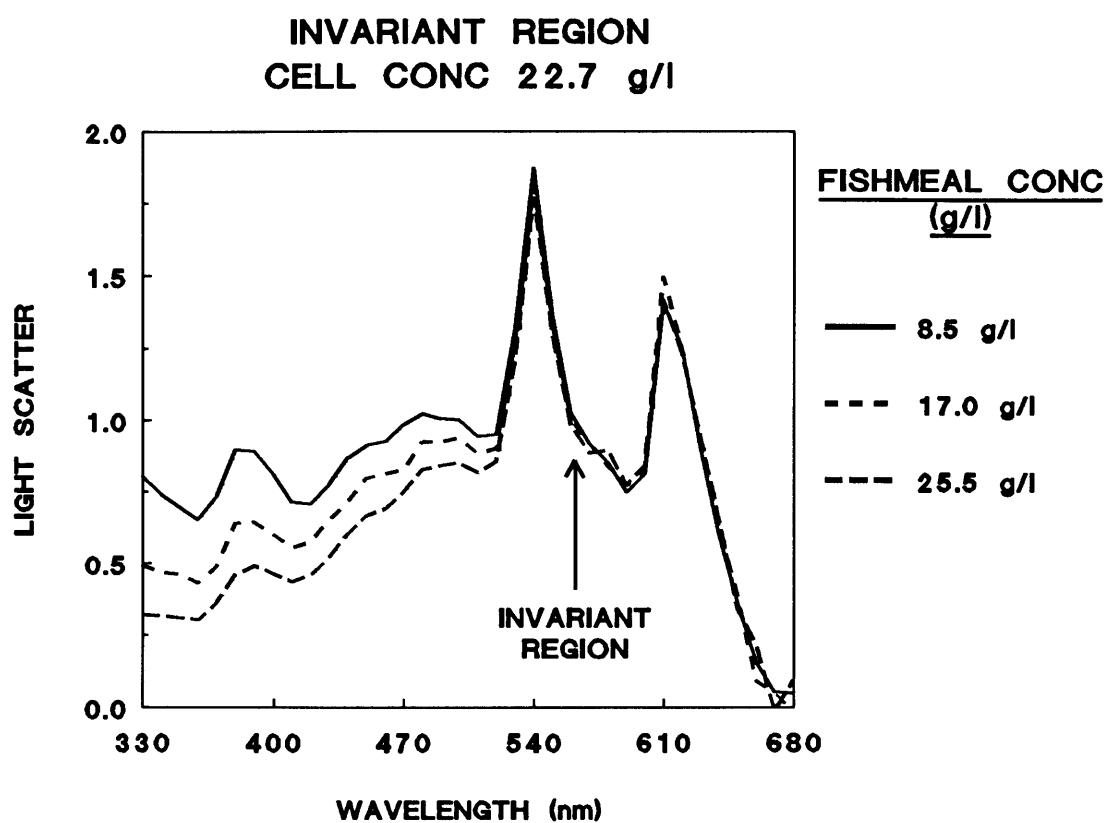


Figure 52: An invariant region in the light scatter spectra of mixtures of *Saccharomyces cerevisiae* and fishmeal at a cell concentration of 22.7 g/l.

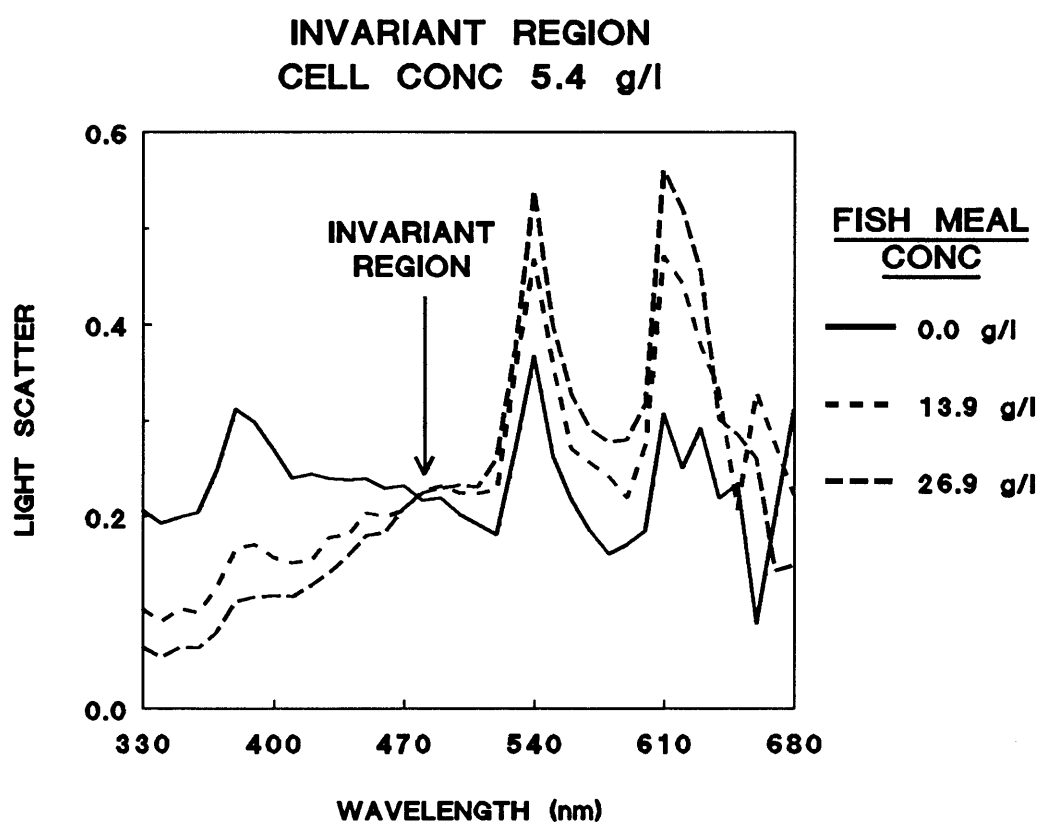


Figure 53: An invariant region in the light scatter spectra of mixtures of *Escherichia coli* and fishmeal at a cell concentration of 5.4 g/l.

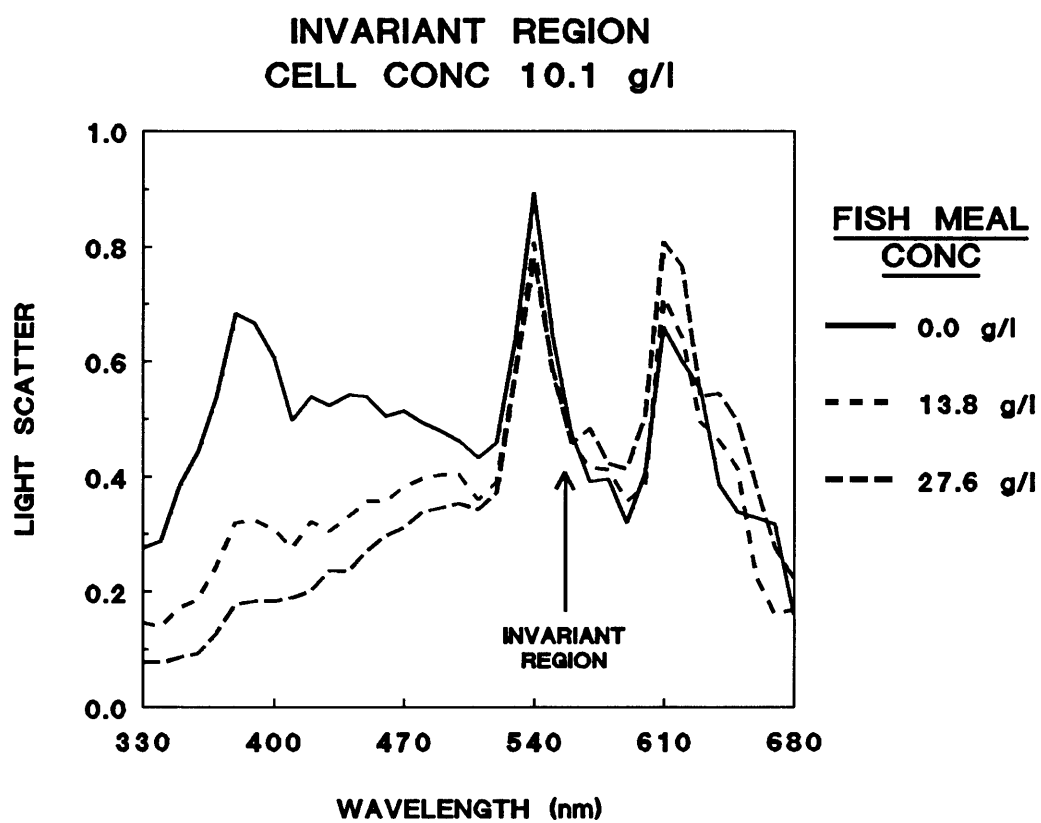


Figure 54: An invariant region in the light scatter spectra of mixtures of *Escherichia coli* and fishmeal at a cell concentration of 10.1 g/l.

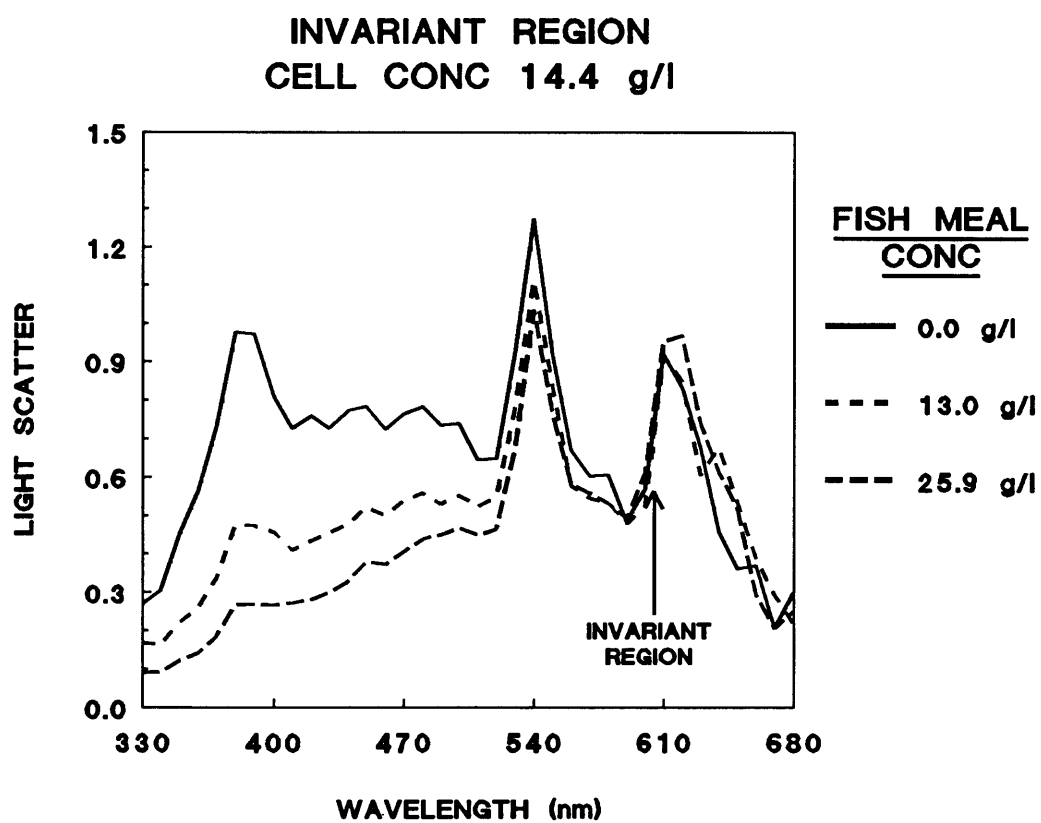


Figure 55: An invariant region in the light scatter spectra of mixtures of *Escherichia coli* and fishmeal at a cell concentration of 14.4 g/l.

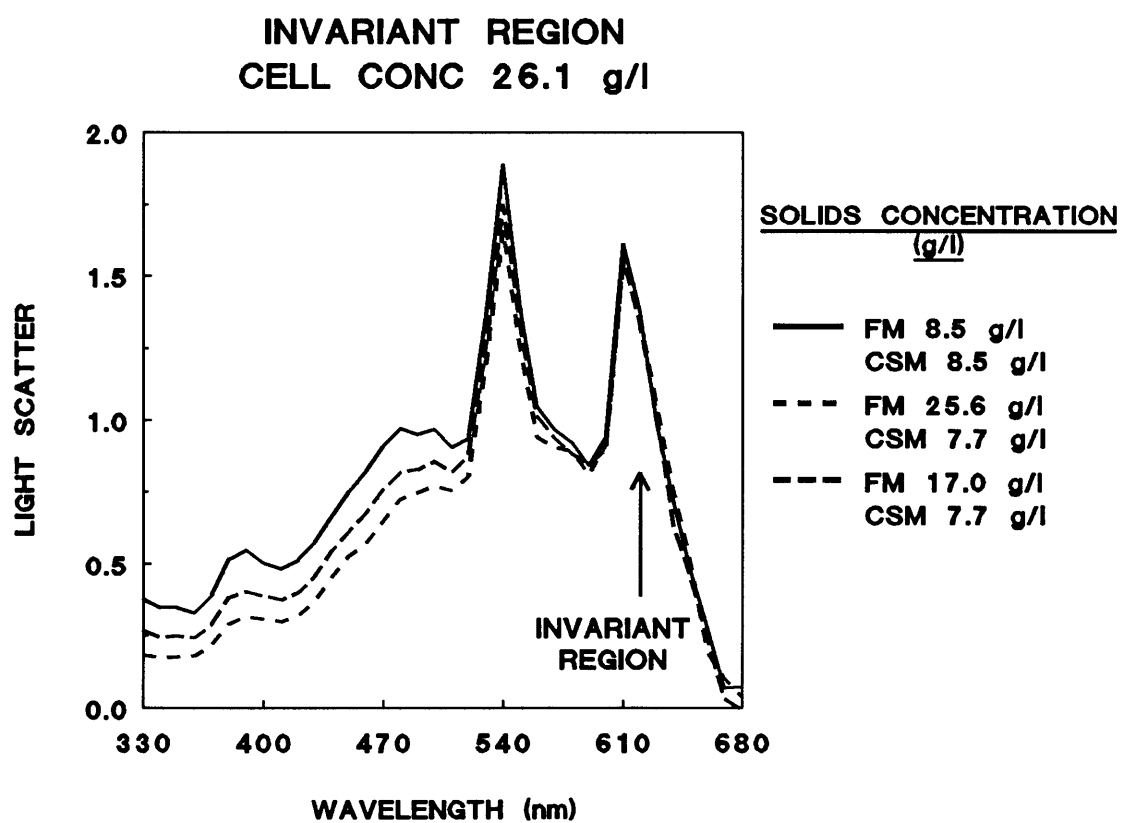


Figure 56: An invariant region in the light scatter spectra of mixtures of *Saccharomyces cerevisiae*, cottonseed meal (CSM) and fishmeal (FM) at a cell concentration of 26.1 g/l.

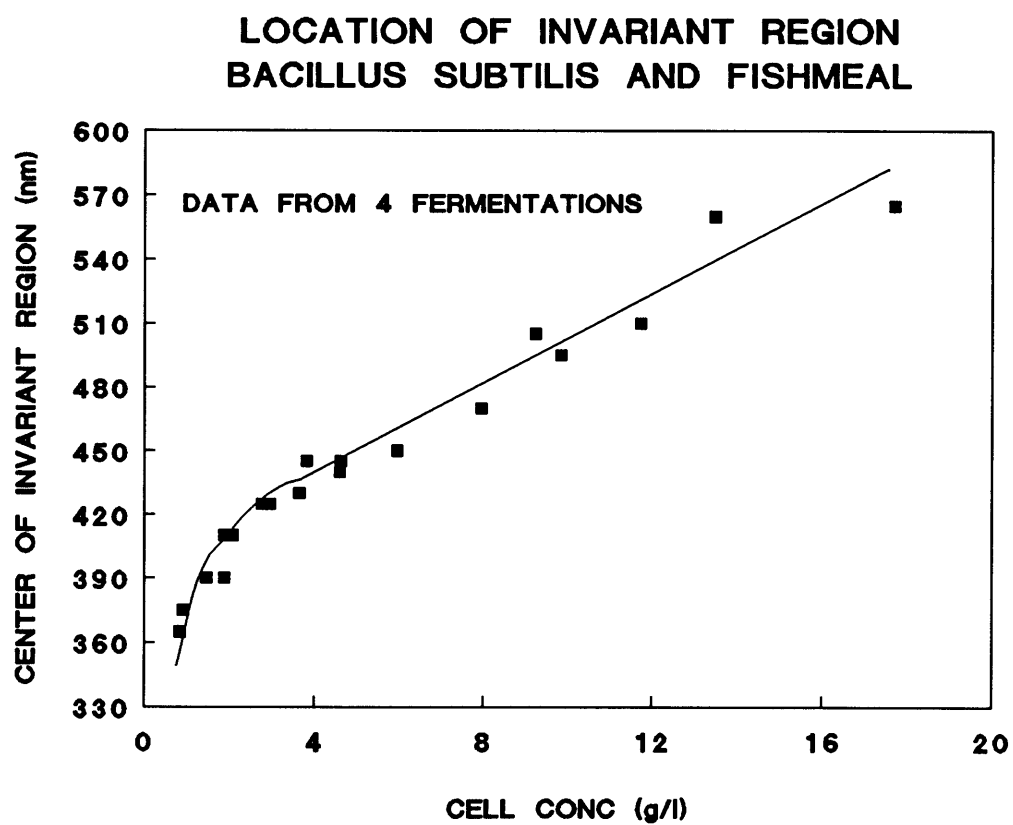


Figure 57: The location of the invariant region as a function of cell concentration for *Bacillus subtilis* and fishmeal.

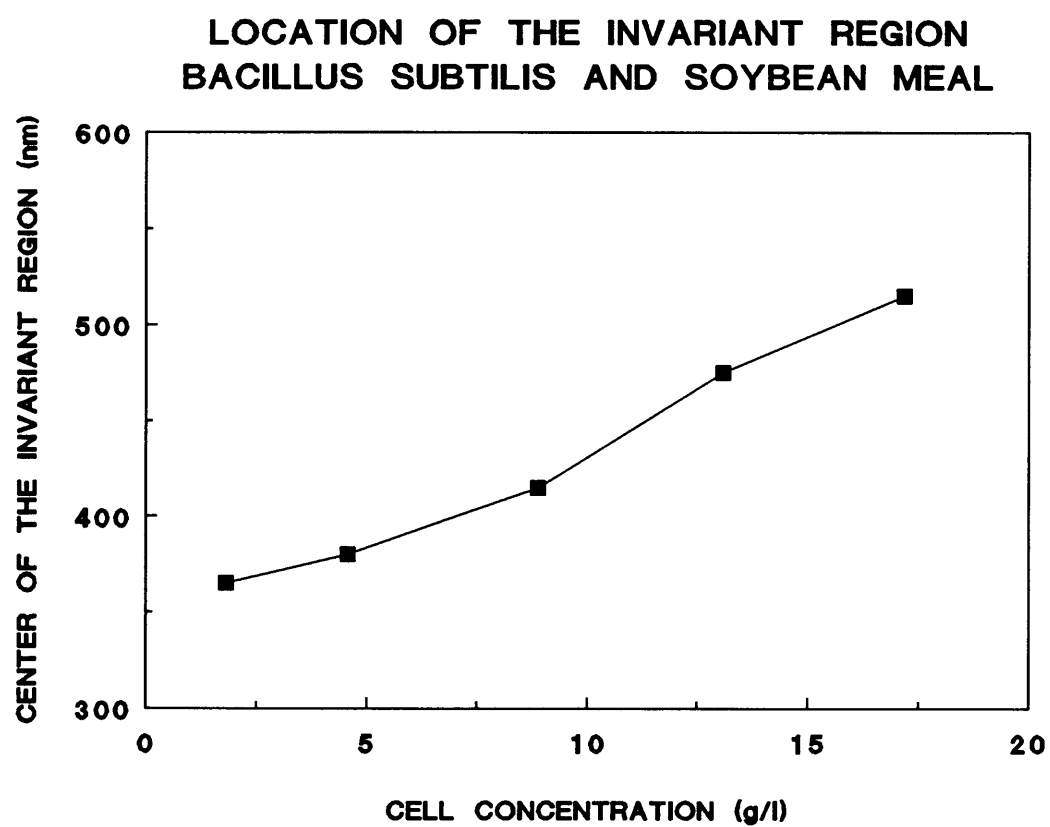


Figure 58: The location of the invariant region as a function of cell concentration for *Bacillus subtilis* and soybean meal.

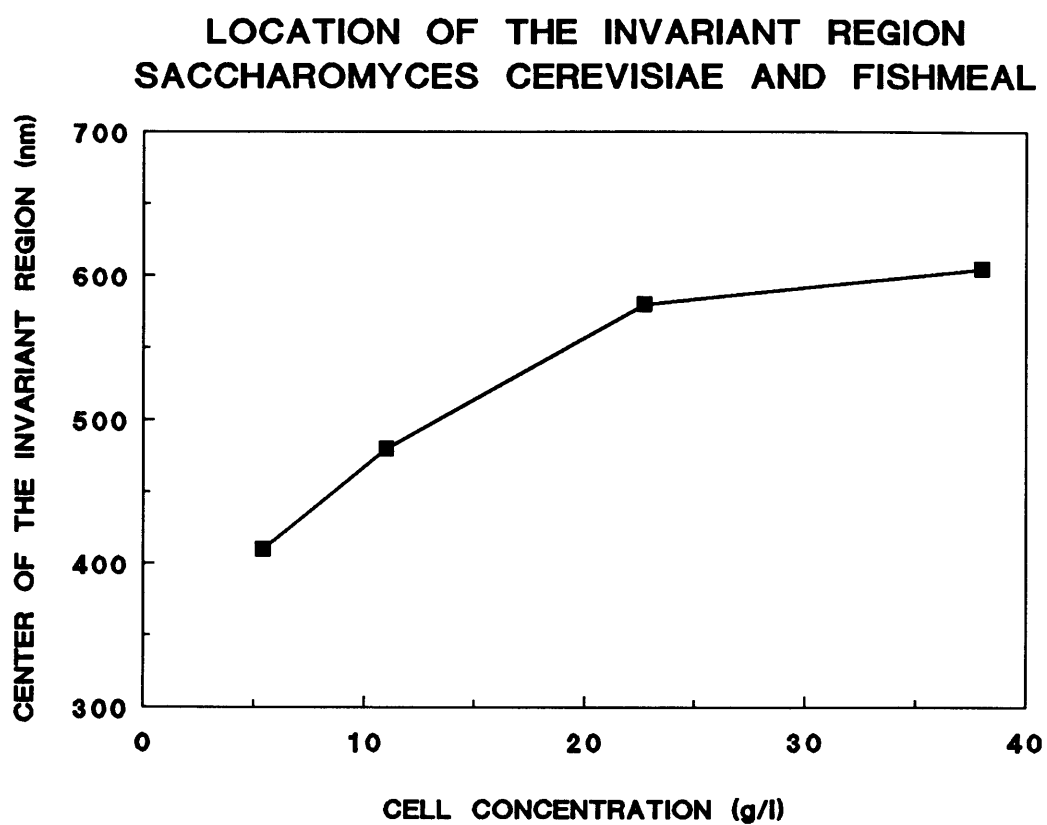


Figure 59: The location of the invariant region as a function of wavelength for *Saccharomyces cerevisiae* and fishmeal.

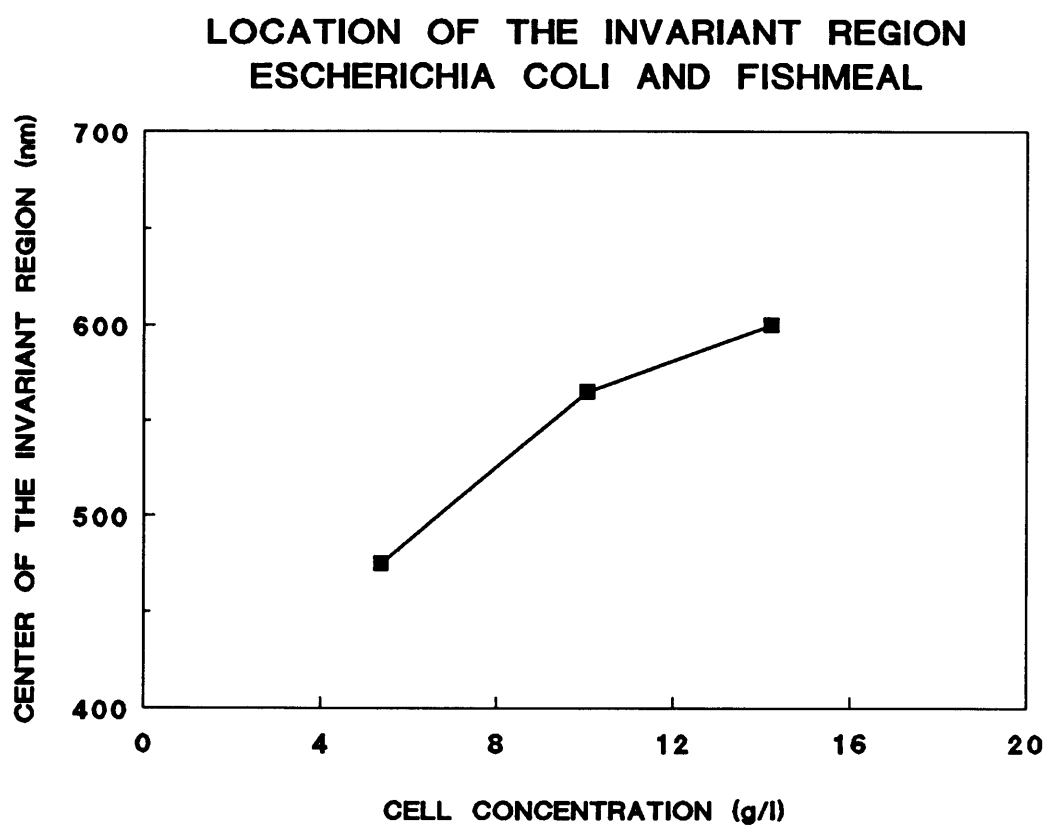


Figure 60: The location of the invariant region as a function of cell concentration for *Escherichia coli* and fishmeal.

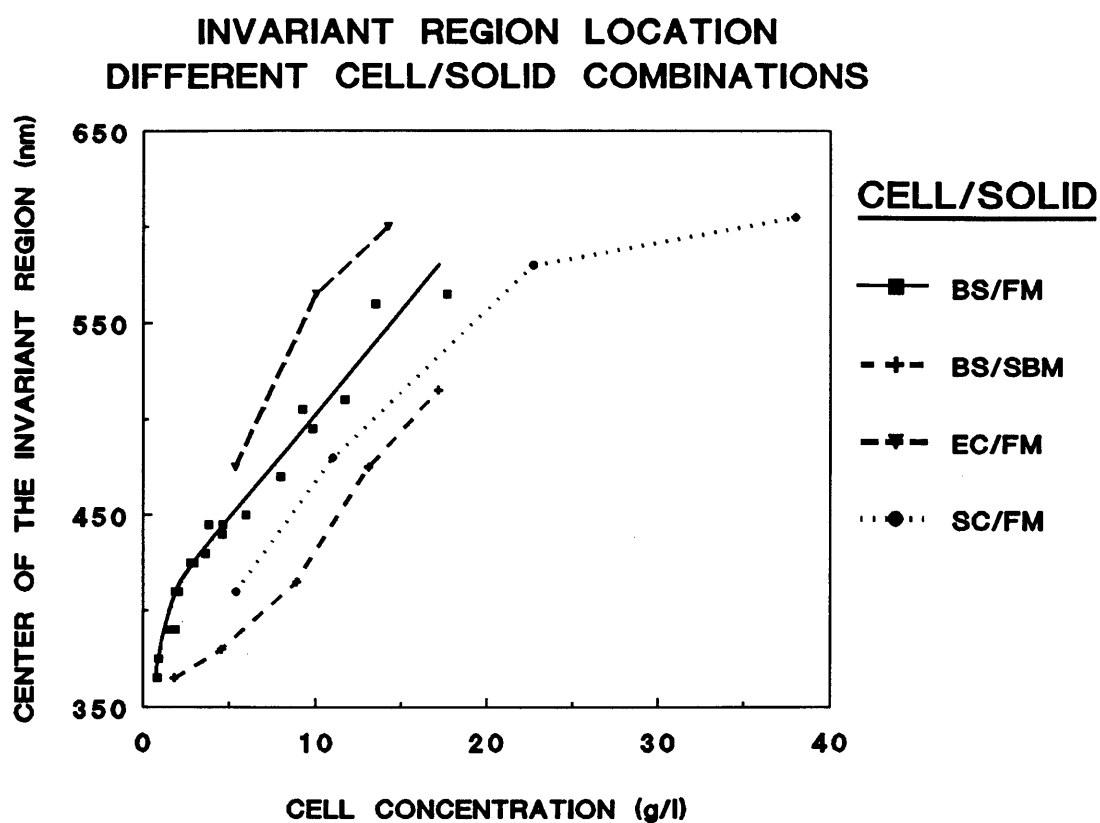


Figure 61: Center of the invariant region as a function of cell concentration for various cell/solid systems. Abbreviations: BS (*Bacillus subtilis*), EC (*Escherichia coli*), SC (*Saccharomyces cerevisiae*), FM (fishmeal), and SBM (soybean meal).

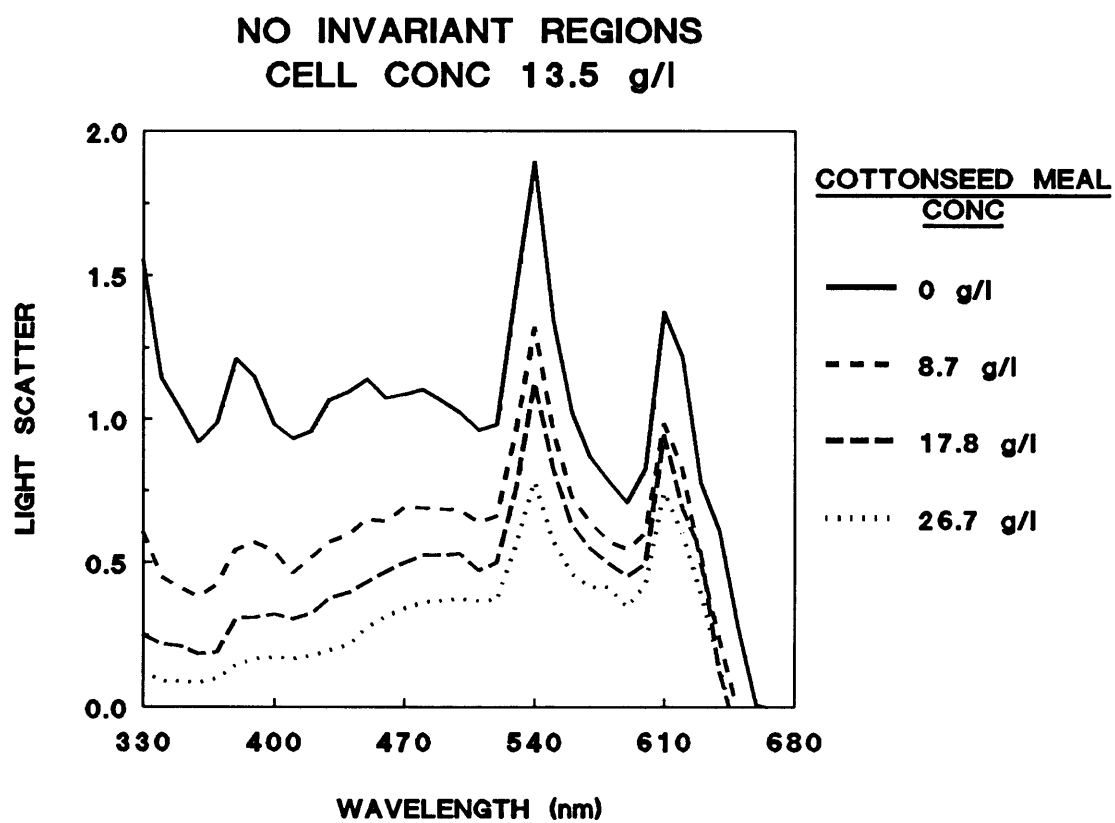


Figure 62: The light scatter spectra of *Bacillus subtilis* and cottonseed meal (180-300 nm). Note the absence of the invariant region.

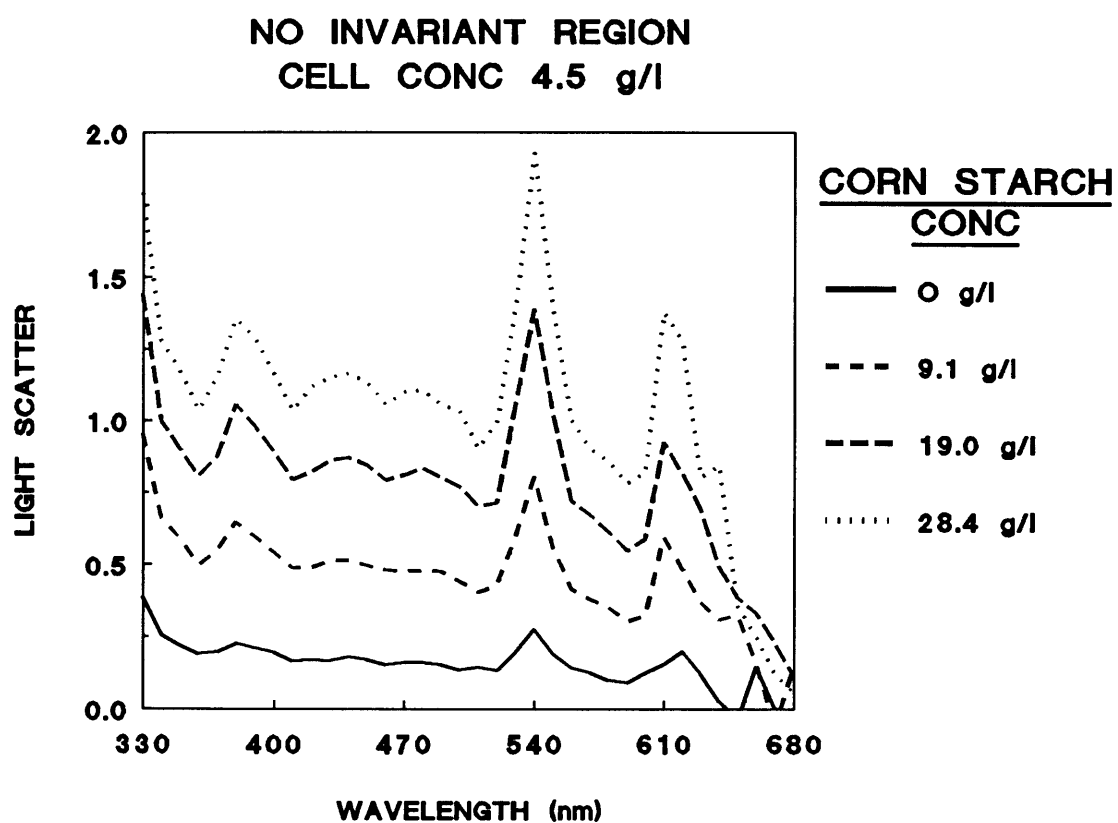


Figure 63: The light scatter spectra of *Bacillus subtilis* and corn starch. Note the absence of the invariant region.

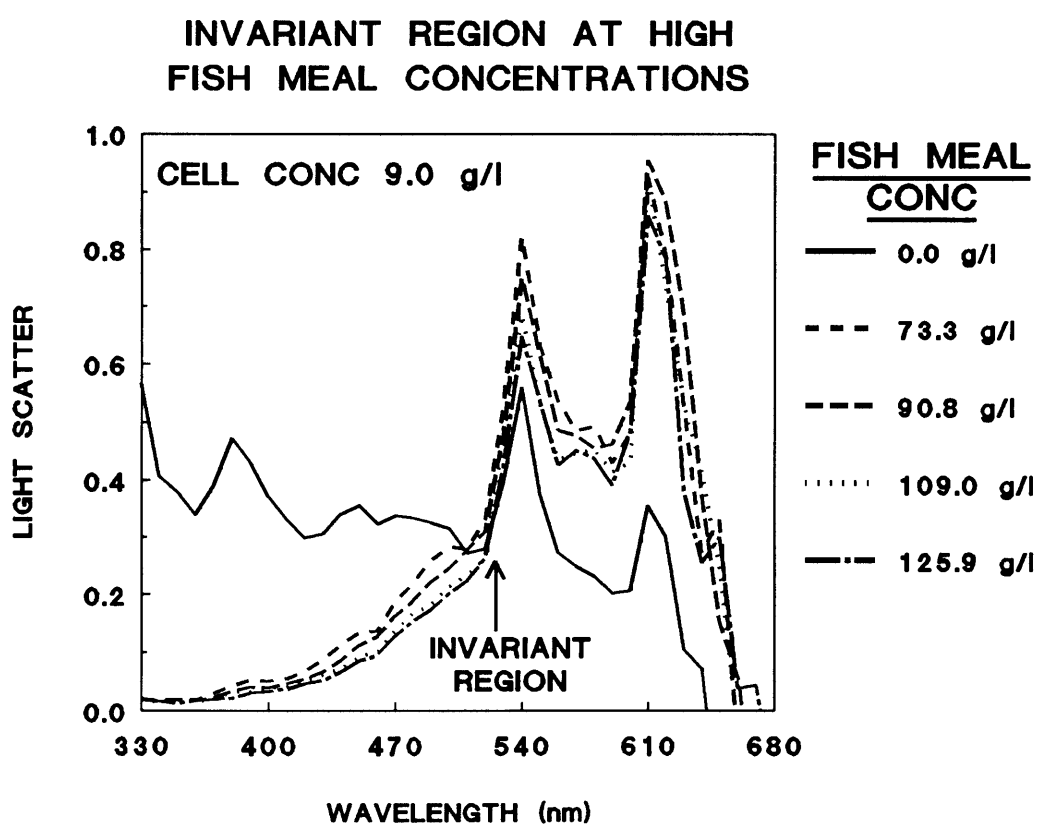


Figure 64: An invariant region in the light scatter spectra of mixtures of *Bacillus subtilis* and fishmeal, at high fishmeal concentrations.

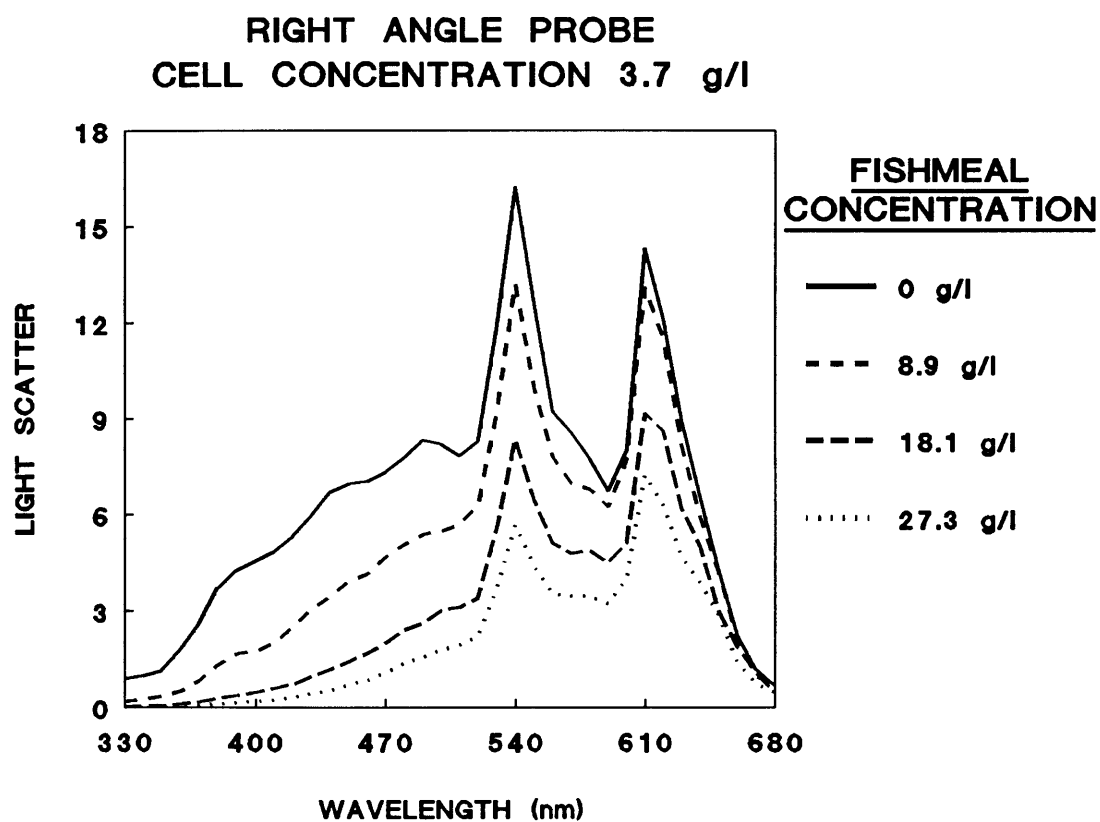


Figure 65: No invariant regions were observed between *Bacillus subtilis* (3.7 g/l) and fishmeal when using the right angle probe.

4.5) The Effect of Particle Size on the Light Scatter Spectrum.

The effect of particle size on the light scatter spectrum is important because the solid substrates will decrease in size during the fermentation. If particle size greatly effects the light scatter spectrum, then it may not be suitable to estimate the cell concentration during fermentation. Experimental evidence, see figures 66 to 68, indicates that the size of the solid substrate does not significantly affect the light scatter spectrum at constant solid concentration. This has been demonstrated for *Saccharomyces cerevisiae*/soybean meal (see figure 66), *Bacillus subtilis*/soybean meal (see figure 67) and *Bacillus subtilis*/fishmeal (see figure 68). The light scatter from *Bacillus subtilis*/fishmeal does show some variation with particle size. A probable cause of this variation is the inhomogeneity of the fishmeal particles. The different particle size fractions were obtained by sieving the whole fishmeal. Particles from different size fractions were of noticeably different composition. No such inhomogeneities were noticeable in the soybean meal size fractions.

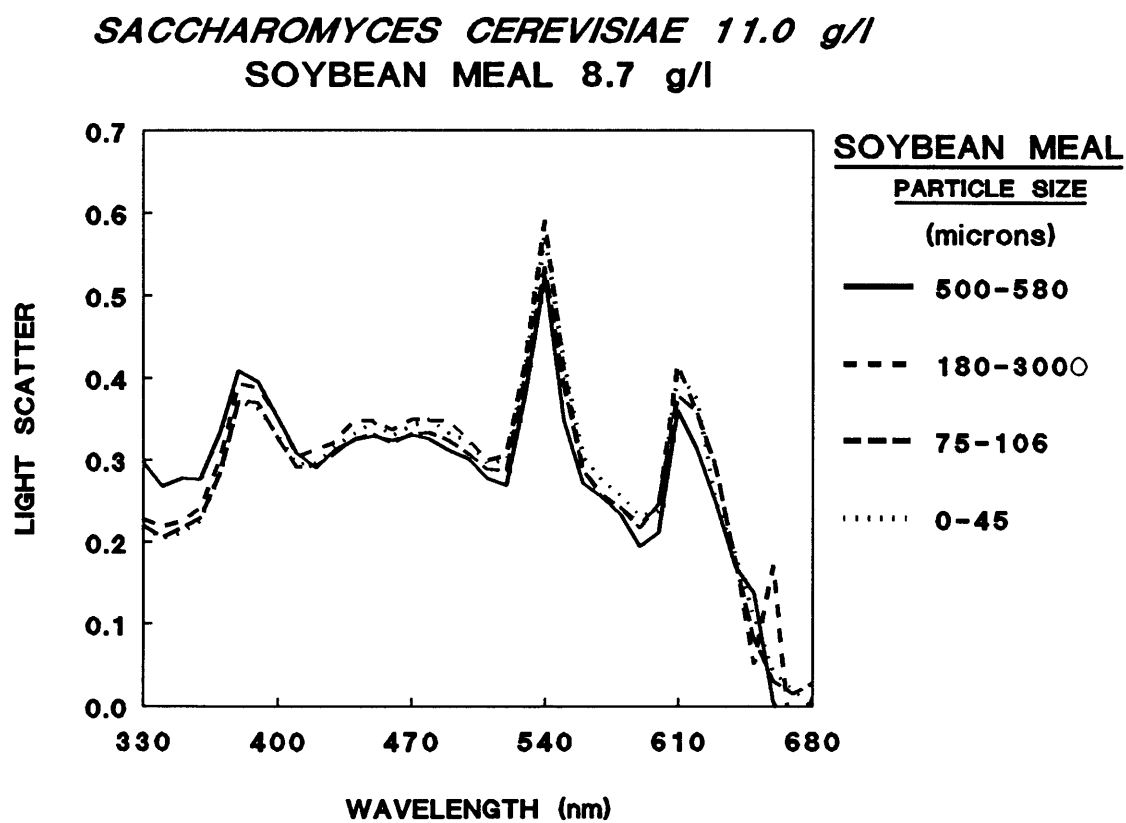


Figure 66: The effect of solid particle size on the light scatter spectrum of *Saccharomyces cerevisiae* and soybean meal.

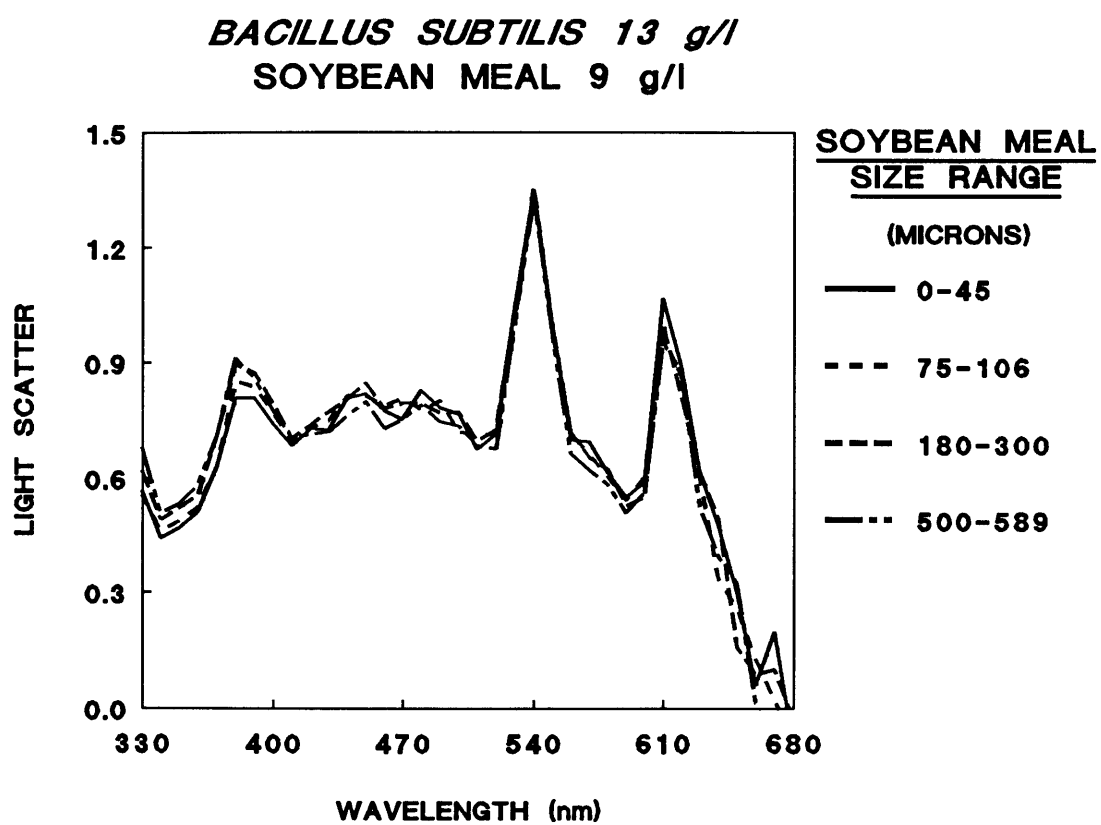


Figure 67: The effect of solid particle size on the light scatter spectrum of *Bacillus subtilis* and soybean meal.

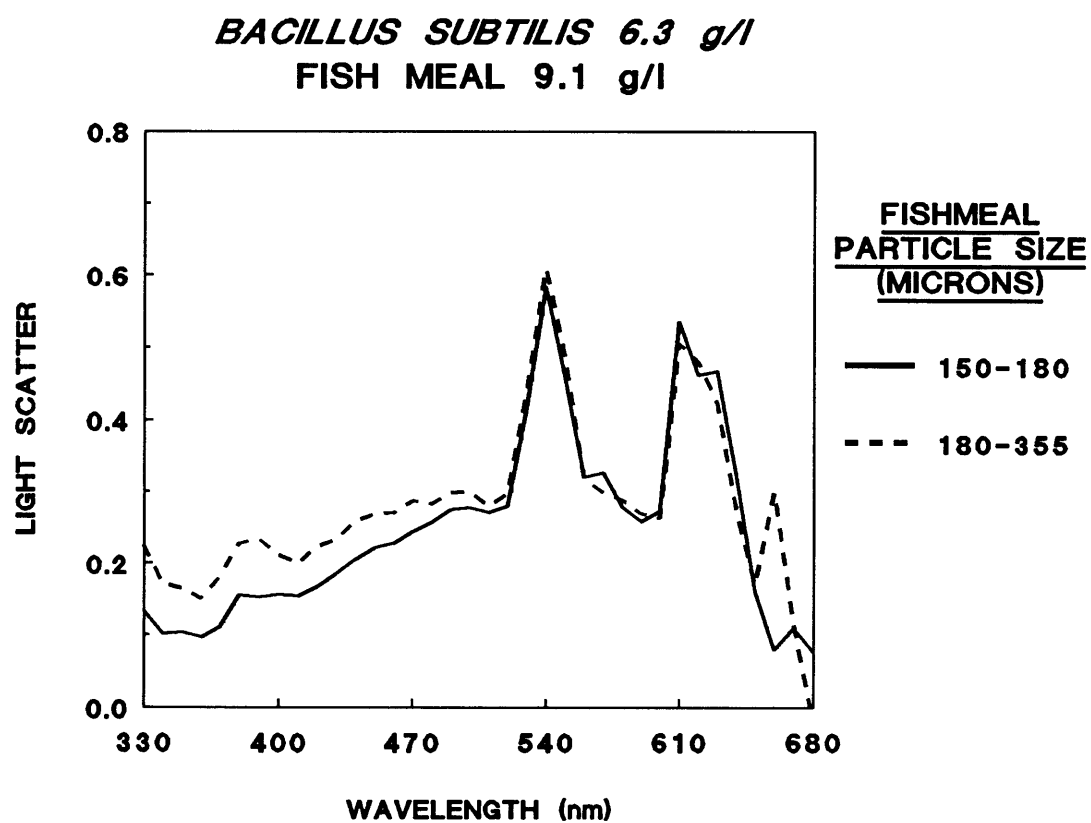


Figure 68: The effect of solid particle size on the light scatter spectrum of *Bacillus subtilis* and fishmeal.

4.6) Estimation of Cell Concentration in the Presence of Solid Substrate using Light Scatter Data

Cell concentration can be estimated during the fermentation by measuring the light scatter spectrum and using two sets of calibration data. Data set one shows the effect of cell concentration on the light scatter spectrum of cells alone, see figure 36. Data set two shows the location of the invariant region as a function of cell concentration, summarized in figure 57. The procedure for estimating the cell concentration is as follows. First locate the invariant region, then using only the invariant region of the light scatter spectrum, estimate the cell concentration using the cell only calibration data, (ie using data set one). This at first appears to be a catch-22 type situation; you cannot find the invariant region until you know the cell concentration, and you cannot estimate the cell concentration until you find the invariant region.

The solution is to scan sequentially the entire light scatter spectrum until the invariant region is found. At each wavelength, using data set one, predict the cell concentration from the fermentation sample light scatter data. Next at each wavelength, using data set two, predict the upper and lower limit of the invariant region in terms of cell concentration. This gives the upper limit and lower limit of the invariant region at each wavelength. If the cell concentration predicted from the fermentation sample is between the upper and lower limits of the invariant region then the cell concentration estimate from the sample is valid. This criterion is applied at each wavelength throughout the light scatter spectrum.

Several consecutive valid cell concentration estimates (ie next to each other in

wavelength) are usually obtained in each fermentation sample light scatter spectrum. These consecutive valid cell concentration estimates are averaged to obtain an overall estimate of the cell concentration. The methodology for estimating the cell concentration can be seen in figure 69. A numerical example can be seen in table 4.

In some spectra, valid cell concentration estimates occurred which were outside a large group of consecutive valid cell concentration estimates. When this situation occurred only the largest group of consecutive valid cell concentration estimates were used to estimate the cell concentration; those outside this region were rejected. These outside points were rejected as they often predicted a cell concentration significantly different than the consecutive valid estimates and thus affected the average. The outside points usually occurred at high wavelengths, where the invariant region was wide.

The light scatter spectrum exhibited a low signal to noise ratio above 620 nm. This low signal to noise ratio lead to inconsistent results above 620 nm, as can be seen for example in figures 45, 47 and 54. Factors such as how well the radiometers were zeroed affected results above 620 nm. For this reason data above 620 nm was not used to predict cell concentration in this study. Thus if the invariant region extended above 620 nm it was not detected.

The cell concentration can also be estimated graphically. At every wavelength along the light scatter spectrum, the cell concentration is estimated using calibration data set one. These estimates of the cell concentration are plotted as a function of the wavelength. Superimposed on top of this plot, with wavelength and cell concentration axes matching respectively, is the location of the invariant region as a function of cell concentration.

Where the cell concentration estimates pass through the invariant region is the valid estimate of cell concentration. An example can be seen in figure 70.

During a fermentation, the cell concentration was estimated every hour and the cell concentration profile is then plotted, from which growth rate data could be extracted. Several techniques were used to estimate the growth rate. A cubic spline could be fitted to the data. The cubic spline is simply a cubic equation fitted to the last three estimates of the cell concentration. The cubic equation is then differentiated and the slope estimated at the most recent time point. This slope (dX/dt) is then used to calculate the growth rate. This worked with reasonable success if a smooth profile of the cell concentration was obtained. If the cell concentration curve was not smooth then this technique produced substantial error.

Another technique, consisting of connecting the last two cell concentration estimates with a straight line, to calculate the slope was also investigated. This technique gave the worst results as the technique only considers the last two data points. The technique that gave the best results was to draw a curve by hand through the data and then manually estimate the slope of this curve. This technique had the advantage that even if the cell concentration profile was not smooth a reasonable value of the growth rate was still obtained.

The change in the light scatter spectrum with time during a fermentation is illustrated in figure 71. Figure 71 is not the actual light scatter spectra, but a distance weighted least squares fit to the data. The data was fitted in this manner to show the trend in light scatter data as a smooth surface rather than a set of points. It is difficult to determine trends in

a set of data points on a three dimensional plot. The data was fitted using SYGRAPH version 1, from Systat Inc. (1800 Sherman Avenue, Evanston, IL 60201).

Validating the light scatter technique was again a catch-22 situation. If there was a good technique available, to compare the light scatter technique to, then we would not have needed to develop the light scatter technique. However, since there was no good absolute technique available, then how could we tell if the light scatter technique was successful? We chose to do this by comparing the light scatter technique to two other techniques - the DNA and the carbon dioxide evolution techniques - in a series of independent fermentations. The DNA and carbon dioxide evolution techniques were chosen because each technique complimented a weakness in the other. The carbon dioxide evolution technique was most accurate at the beginning of the fermentation and the DNA technique was most accurate at the end of the fermentation, see sections 3.2.1 and 3.2.2. This gave us some correct coverage for the entire fermentation.

The cell concentration estimated using the light scatter technique was compared to DNA and carbon dioxide evolution estimates of the cell concentration in three separate solid substrate fermentations, see figures 72, 73 and 74. Keep in mind when looking at these figures that the DNA and carbon dioxide evolution techniques contain errors. Also, it can be seen in figures 72-74 that the light scatter technique significantly overestimates the cell concentration, compared to the other two techniques, when the cell concentration is below 4.5 g/l.

There are two probable causes of the discrepancy in the light scatter data at low cell concentrations (below 4.5 g/l). The first probable cause is that the morphology changes

during this part of the fermentation. During the early part of the fermentation, when the cell concentration is low, the cells form chains. At the end of the fermentation the cells exhibit a single cell morphology. All the calibration data used in this study was generated from cells in a single cell morphology. Thus a changing cell morphology may affect the light scatter technique.

The second probable cause of the discrepancy is that the slope of the location of the invariant region as a function of cell concentration is very steep below 4.5 g/l, see figure 57. This means that below 4.5 g/l the invariant region is more difficult to locate as a small change in cell concentration means a large change in the position of the invariant region.

The light scatter technique was correctly able to follow cell concentration (as predicted by the DNA technique) as the culture entered the stationary phase, see figure 72. Thus the light scatter technique was able to overcome one of the main weaknesses of the carbon dioxide evolution technique, namely the inability to identify the stationary phase. The light scatter technique was also able to overcome one of the main weakness of the DNA technique, namely that the DNA technique is a very slow off-line assay.

It is possible that there is a limit to the cell concentration that can be measured using this technique. This limitation would occur if the invariant region moved above 680 nm and thus could not be measured using the equipment in this study. It is unclear if this will be a problem, as figure 59 shows that at higher cell concentrations (above 20 g/l) the location of the invariant region is not strongly affected by cell concentration.

The results of the light scatter technique from four independent solid substrate fermentations were compared to the average of the carbon dioxide evolution and DNA

techniques. Light scatter cell estimates below 4.5 g/l were not included in the analysis because the light scatter technique is known to fail under these conditions. The average difference between the light scatter technique and the average of the carbon dioxide evolution and DNA techniques was +0.9 g/l. The standard deviation of this difference was 2.4 g/l. For comparison, in solid free fermentations, the average difference between the carbon dioxide evolution technique and a dry cell weight measurement was 0.0 g/l, with the standard deviation of the difference equal to 0.9 g/l. Also in a solid free fermentation, the average difference between the DNA technique and a dry cell weight measurement was 0.1 g/l, with the standard deviation of the difference equal to 1.2 g/l.

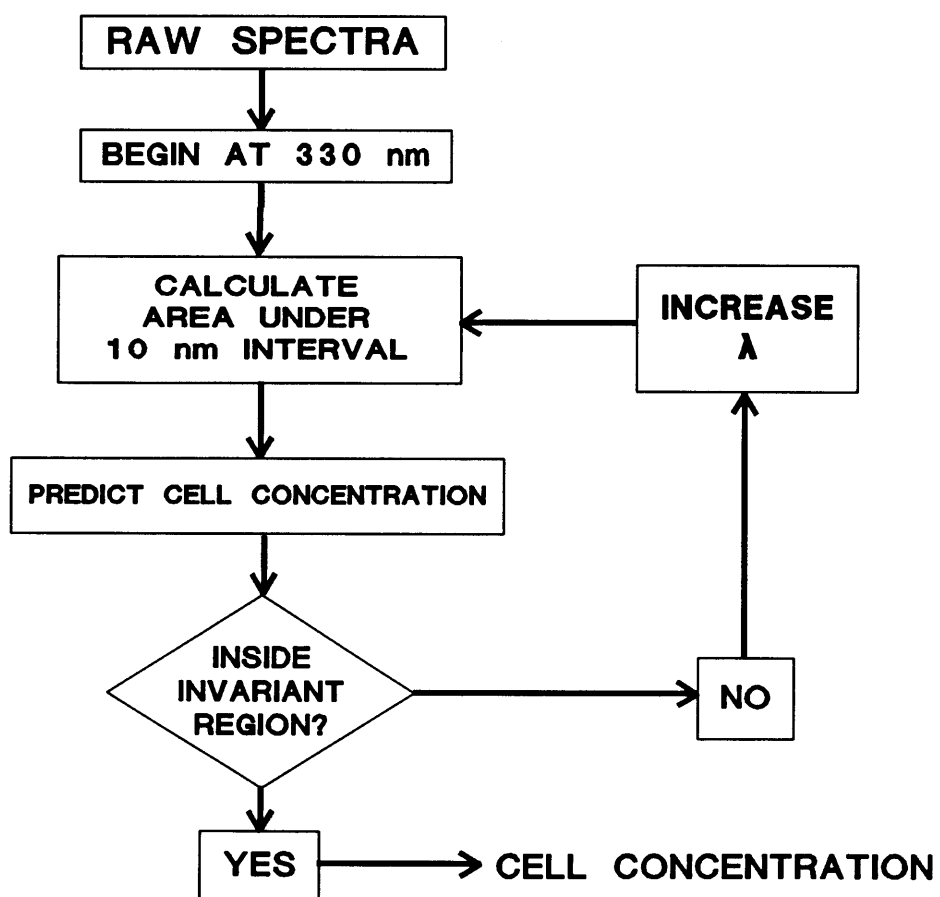


Figure 69: The methodology used to calculate cell concentration from light scatter data.

table 4: An example calculation taken from fermentation data of the growth of *Bacillus subtilis* on fishmeal.

WAVELENGTH RANGE (nm)	ILS	PREDICTED CELL CONC (g/l)	IR LOWER LIMIT (g/l)	IR UPPER LIMIT (g/l)	INSIDE IR ? (Y/N)	PREDICTED SOLUTION
330-340	0.74353	1.2	0.2	0.8		
340-350	0.68034	1.3	0.3	0.9		
350-360	0.74032	1.6	0.4	1.1		
360-370	0.86082	1.9	0.5	1.3		
370-380	1.14392	2.2	0.6	1.5		
380-390	1.39747	2.5	0.8	1.8		
390-400	1.48038	2.9	1.0	2.0		
400-410	1.48925	3.0	1.3	2.3		
410-420	1.63711	3.4	1.6	2.8		
420-430	1.93966	3.9	2.1	3.3		
430-440	2.21464	4.2	2.6	4.0		
440-450	2.45246	4.5	3.2	5.0	YES	4.5
450-460	2.68806	5.0	3.9	6.0	YES	5.0
460-470	3.09343	5.7	4.8	7.0	YES	5.7
470-480	3.59394	6.4	5.7	8.3	YES	6.4
480-490	3.86856	7.0	6.6	9.4	YES	7.0
490-500	4.00751	7.5	7.5	10.4		
500-510	4.01334	7.8	8.3	11.5		
510-520	4.12256	8.3	9.2	12.6		
520-530	5.37025	8.7	10.0	13.7		
530-540	7.80188	9.1	10.8	14.8		
540-550	7.84820	9.3	11.7	15.8		
550-560	5.89563	9.6	12.6	16.9		
560-570	4.99100	11.1	13.4	18.0		
570-580	4.69936	11.2	14.2	19.1		
580-590	4.39224	11.0	15.1	20.1		
590-600	4.27427	11.2	15.9	21.2		
600-610	6.47512	12.2	16.7	22.3		
610-620	7.91864	12.6	17.5	23.4		
AVG						5.7

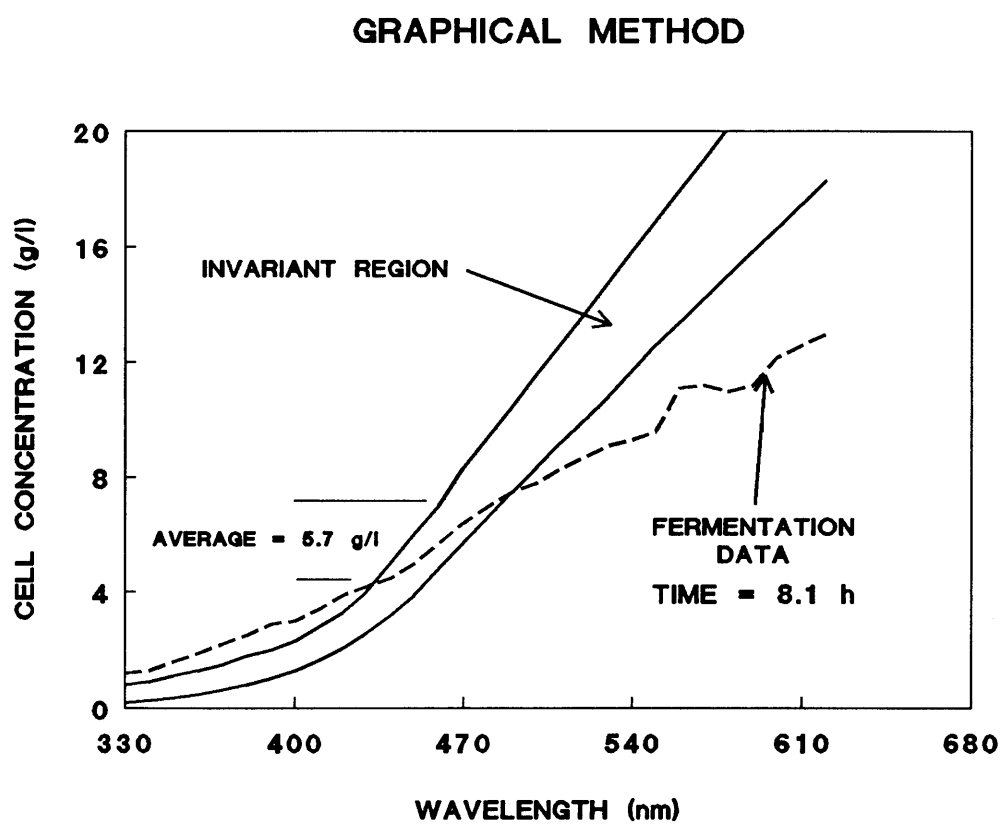


Figure 70: The graphical method of estimating cell concentration.

FERMENTATION DATA

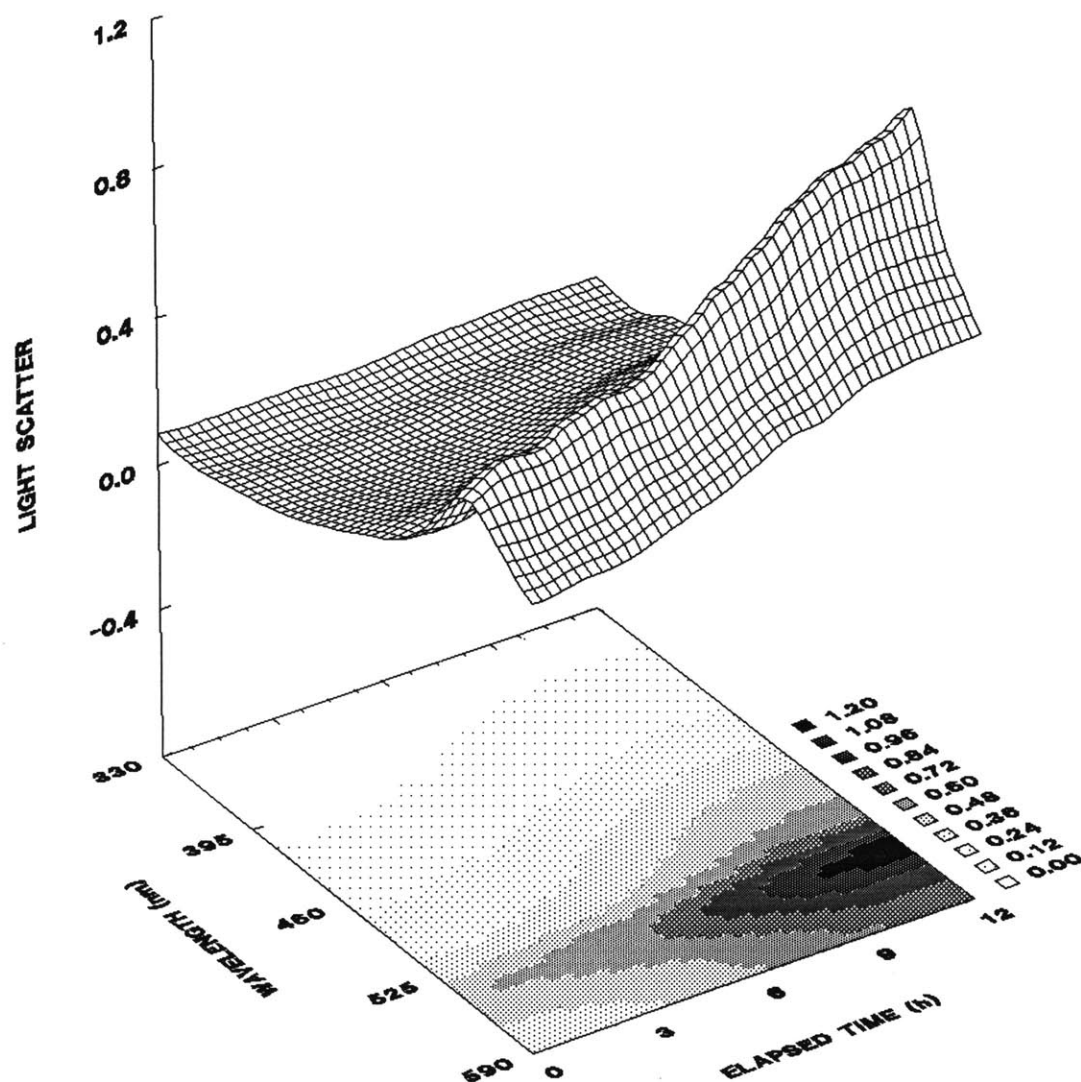


Figure 71: The light scatter spectra obtained during a fermentation. The third dimension represents the time during the fermentation.

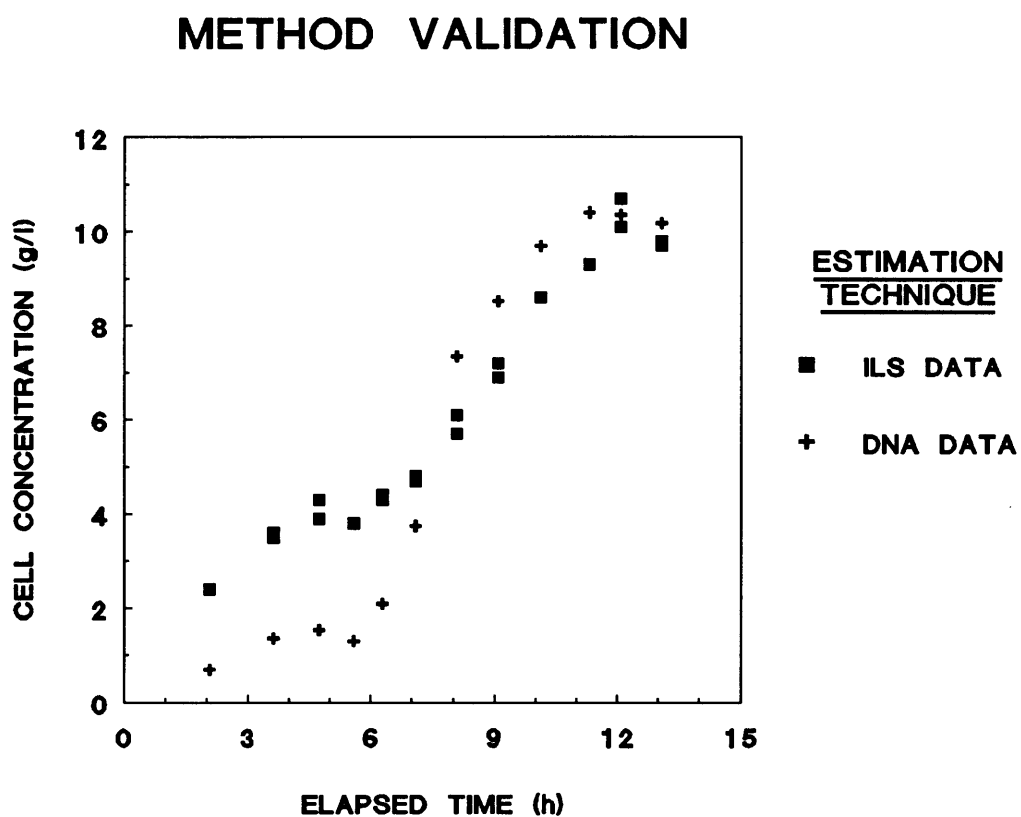


Figure 72: Comparison of cell concentration predictions from the light scatter technique with those from the DNA technique during the growth of *Bacillus subtilis* on fishmeal.

METHOD VALIDATION

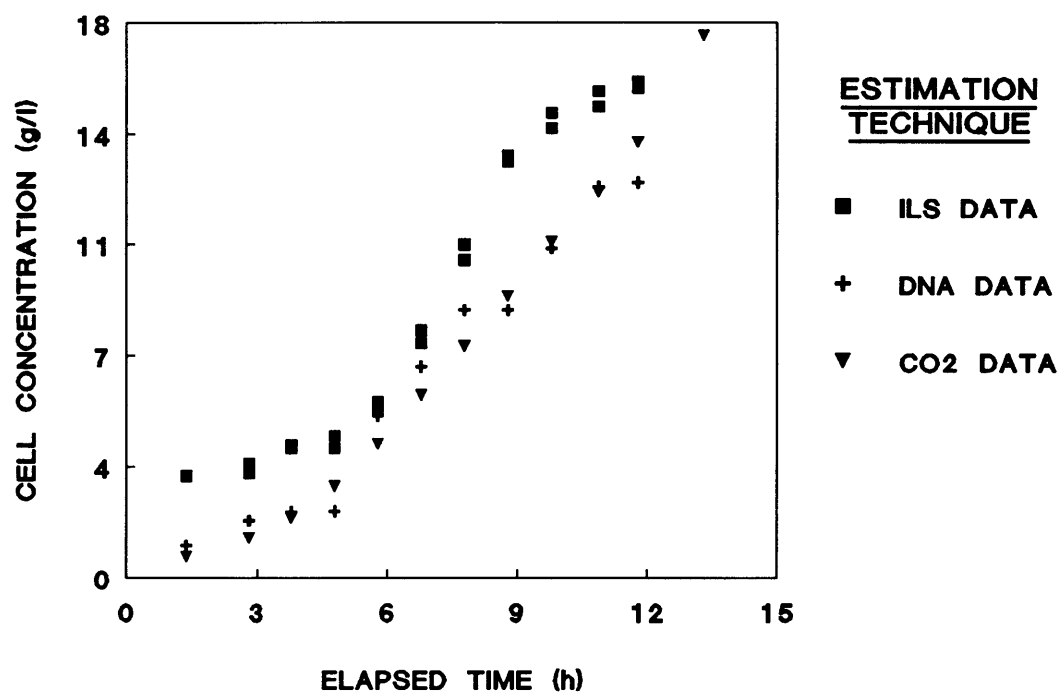


Figure 73: Comparison of cell concentration predictions from the light scatter technique with those from the DNA and carbon dioxide evolution techniques during the growth of *Bacillus subtilis* on fishmeal.

METHOD VALIDATION

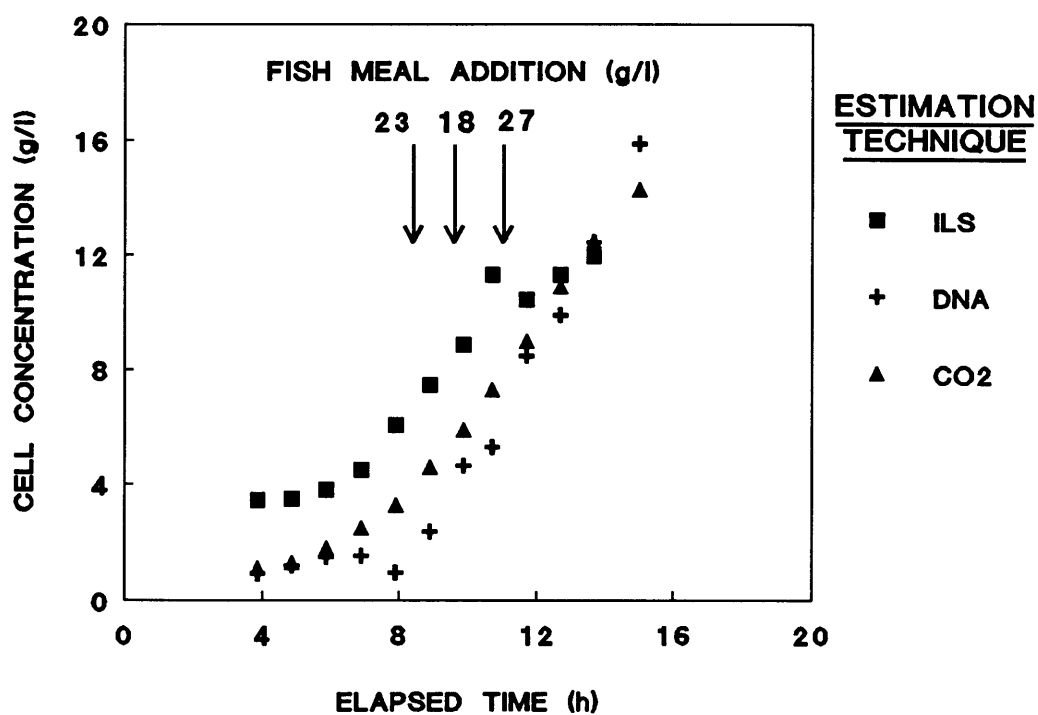


Figure 74: Comparison of cell concentration predictions from the light scatter technique, with those from the DNA and carbon dioxide evolution techniques during the fed-batch fermentation of *Bacillus subtilis* on fishmeal.

4.7) Estimation of Low Cell Concentrations.

The light scatter technique overestimates the cell concentration below 4-5g/l. One possible solution to this problem is to make use of an independent, but known cell concentration estimate. One such independent estimate is the cell concentration at the beginning of the fermentation. The cell concentration at the beginning of the fermentation can be estimated from the cell concentration in the inoculum, if this is known, or from data of previous identical inoculum development processes.

The principle of the correction technique is to use the independent estimate of the cell concentration to pinpoint the location of the invariant region. This wavelength is pinpointed by finding the wavelength of the measured light scatter spectrum which correctly predicts the independently estimated cell concentration. The location of the invariant region, based on the independent estimate of cell concentration, is at a lower wavelength than that determined in the usual manner. This gives a $\Delta\lambda$ value for the cell concentration predicted using the normal technique. The $\Delta\lambda$ value is the difference between the location of invariant region determined using the normal technique and that determined from the independent cell mass estimate. This $\Delta\lambda$ value is zero at 4.5 g/l since at this cell concentration the light scatter technique predicts the cell concentration correctly. A straight line is then fitted between these two points of $\Delta\lambda$. This gives $\Delta\lambda$ as a function of cell concentration determined using the normal technique.

To use the correction technique the cell concentration is predicted from the light scatter technique in the normal fashion. Using the straight line relationship between $\Delta\lambda$ and cell concentration, a value of $\Delta\lambda$ is obtained. A new invariant region location is then

calculated. The new invariant region location, is the location of the invariant region determined in the usual manner, minus the $\Delta\lambda$ value. Once the new invariant region has been determined the cell concentration is determined at that wavelength using data set one. The correction technique described above is not used above 4.5 g/l.

To illustrate the correction technique the data from two fermentations were corrected using the initial or second DNA estimate of the cell concentration as the independent measure of cell concentration. The improved cell concentration profiles, obtained using the above technique can be seen in figures 75 and 76.

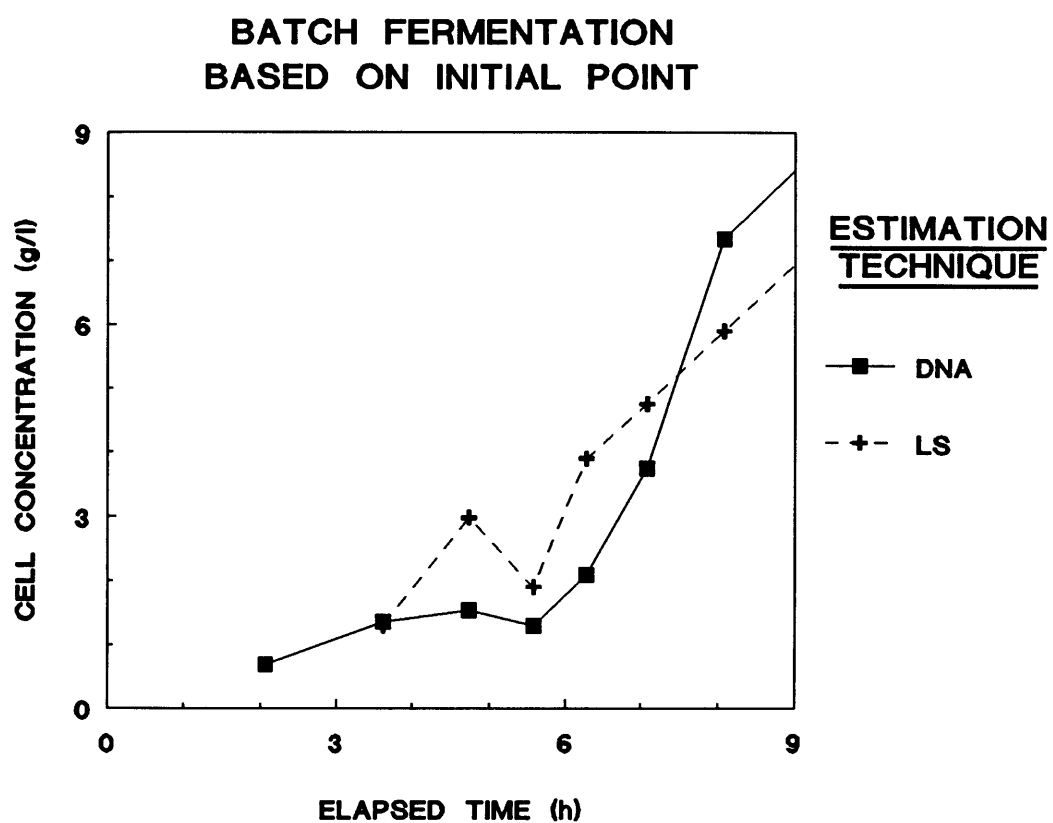


Figure 75: The cell concentration obtained by correcting the light scatter data, compared with the DNA technique.

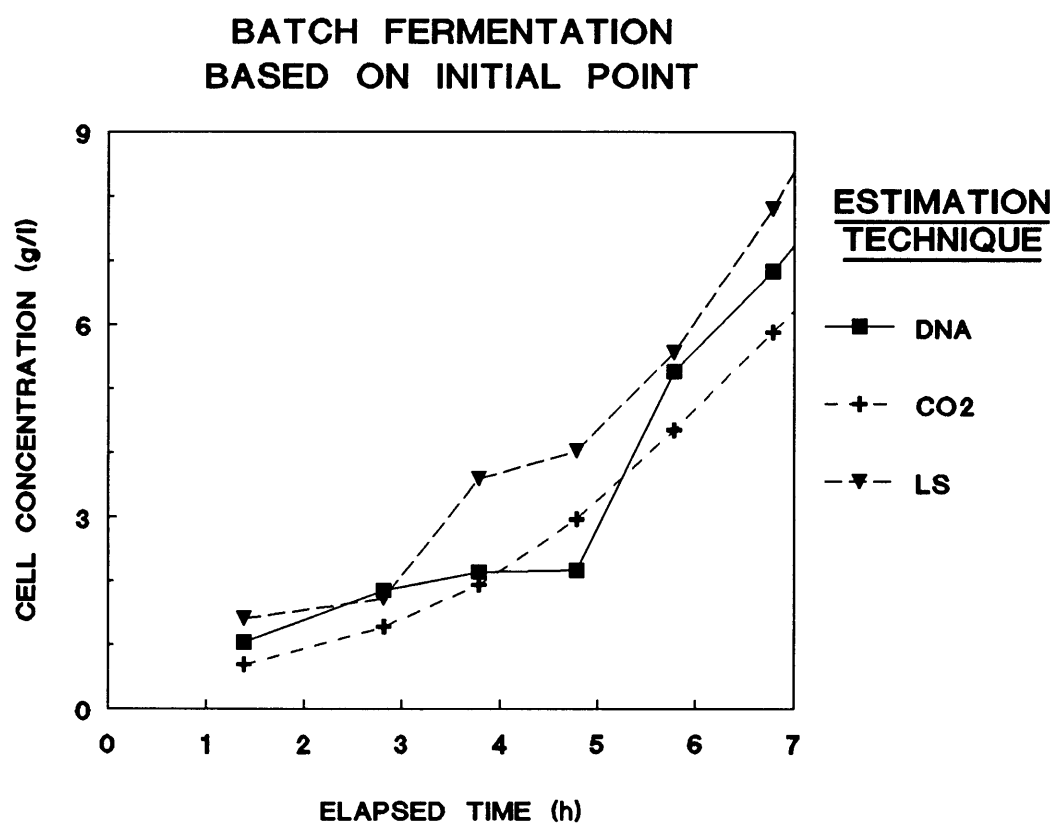


Figure 76: The cell concentration obtained by correcting the light scatter data, compared with the DNA and carbon dioxide evolution techniques.

4.8) A Model Describing the Light Scatter Spectrum of Cell and Solid Mixtures

The aims of this model are as follows. First to investigate the interactions that make up the light scatter spectrum of cells and solids. Second to find evidence to support the hypothesis that the invariant regions are caused by a change in the absorbance of the solid substrate as a function of wavelength. A schematic diagram of the model can be seen in figure 77. The model assumes that both the cells and the solid scatter and absorb light.

The following interaction model was proposed.

$$\begin{aligned}
 L = & \frac{\text{SCATTER FROM THE CELLS}}{\left[\frac{\text{FRACTION ABSORBED BY CELLS}}{1 - \text{FRACTION ABSORBED BY CELLS}} \right]} \cdot \left[\frac{\text{FRACTION ABSORBED BY SOLIDS}}{1 - \text{FRACTION ABSORBED BY SOLIDS}} \right] \\
 & + \frac{\text{SCATTER FROM THE SOLIDS}}{\left[\frac{\text{FRACTION ABSORBED BY SOLIDS}}{1 - \text{FRACTION ABSORBED BY SOLIDS}} \right]} \cdot \left[\frac{\text{FRACTION ABSORBED BY CELLS}}{1 - \text{FRACTION ABSORBED BY CELLS}} \right]
 \end{aligned} \tag{12}$$

where:

L : Light scatter reading

The first two terms in equation 12, the scatter from the cells and the absorbance of this scatter by the cells themselves, is what is measured if we measure the cell only spectrum. The fourth and fifth terms in equation 12, the scatter from the solid and the absorbance of this scatter by the solids themselves, is what is measured if we measure the solid only spectrum. These pure component spectra were thus substituted for the corresponding terms in equation 12.

The Beer-Lambert law, see equation (1), was used to describe the absorption process.

The following assumptions were made.

Assumptions:

- 1) The path length is small (< 1 mm) so that the Beer-Lambert law applies at high concentrations.
- 2) The path length is constant.
- 3) The extinction coefficient is not a function of concentration.
- 4) Since the parameters, ϵ the extinction coefficient and B the path length were not known for this system they were grouped together to form the "absorbance index" as follows

$$ABSORBANCE\ INDEX\ (Q) - \epsilon \cdot B \quad (13)$$

The absorbance index is a measure of the absorbance capacity of the system. A high absorbance index indicates that a large amount of absorbance is occurring and a low absorbance index indicates that not much absorbance is occurring.

Substituting the Beer-Lambert law and assumptions into the interaction model gives the following expression.

$$L - f(\lambda, X_C) \cdot e^{-X_S Q_S} + g(\lambda, X_S) \cdot e^{-X_C Q_C} \quad (14)$$

where:

- λ : wavelength
- X_C : cell concentration
- X_S : solid concentration
- Q_C : cell absorbance index
- Q_S : solid absorbance index
- $f(\lambda, X_C)$: cell only light scatter spectrum, (function of wavelength and cell concentration)

$g(\lambda, X_s)$: solid only light scatter spectrum, (function of wavelength and solid concentration)

Equation 14 was fitted to a series of light scatter data of cell/solid mixtures. The data was fitted using a non-linear regression package, STATGRAPHICS version 2.1 (Statistical Graphics Corporation, 2115 East Jefferson Street, Rockville, Maryland 20852). Data of varying wavelength, and cell and solid concentration was fitted to the model. The inputs to the model were the cell only spectra, the solid only spectra and the spectra of defined mixtures. The output from the model was the fitted absorbance parameters Q_s and Q_c . The model gave a good fit to a large amount of data, see figure 78.

Next the model was used to predict the light scatter spectrum of a series of cell and solid concentrations at various wavelengths. This served as an internal check of the model - the model was used to predict part of the data from which it was derived. The model correctly predicted the light scatter spectrum even though cell and solid concentrations and wavelength varied widely, see figures 79 and 80.

The model was then used to evaluate the solid absorbance index as a function of wavelength. The solid absorbance index is a strong function of wavelength, see figure 81. The solid absorbance index was greatest at low wavelengths and decreased as the wavelength increased. The absorbance index being low at high wavelengths supported the hypothesis that scatter dominates at high wavelengths. The absorbance index being high at low wavelengths supported the hypothesis that absorbance dominates at low wavelengths. The change in absorbance index with wavelength is consistent with the hypothesis that the invariant regions are caused by changes in the absorbance of the solid substrate as a

function of wavelength.

MODEL OF LIGHT SCATTER DATA

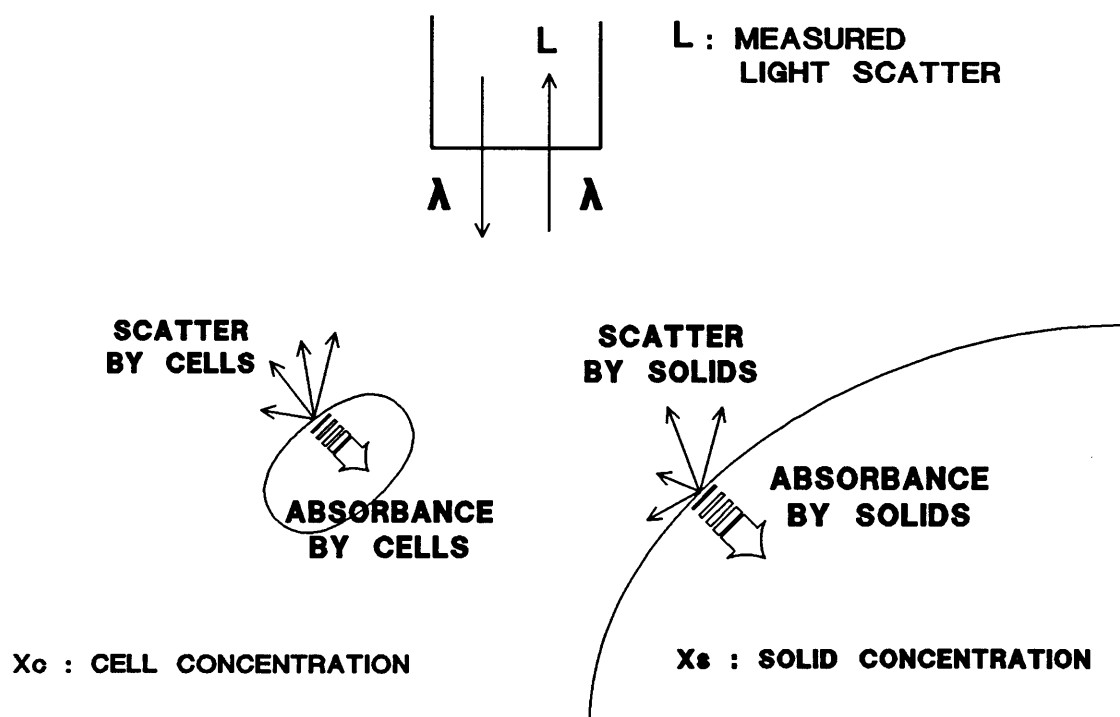


Figure 77: Schematic diagram of the light scatter model.

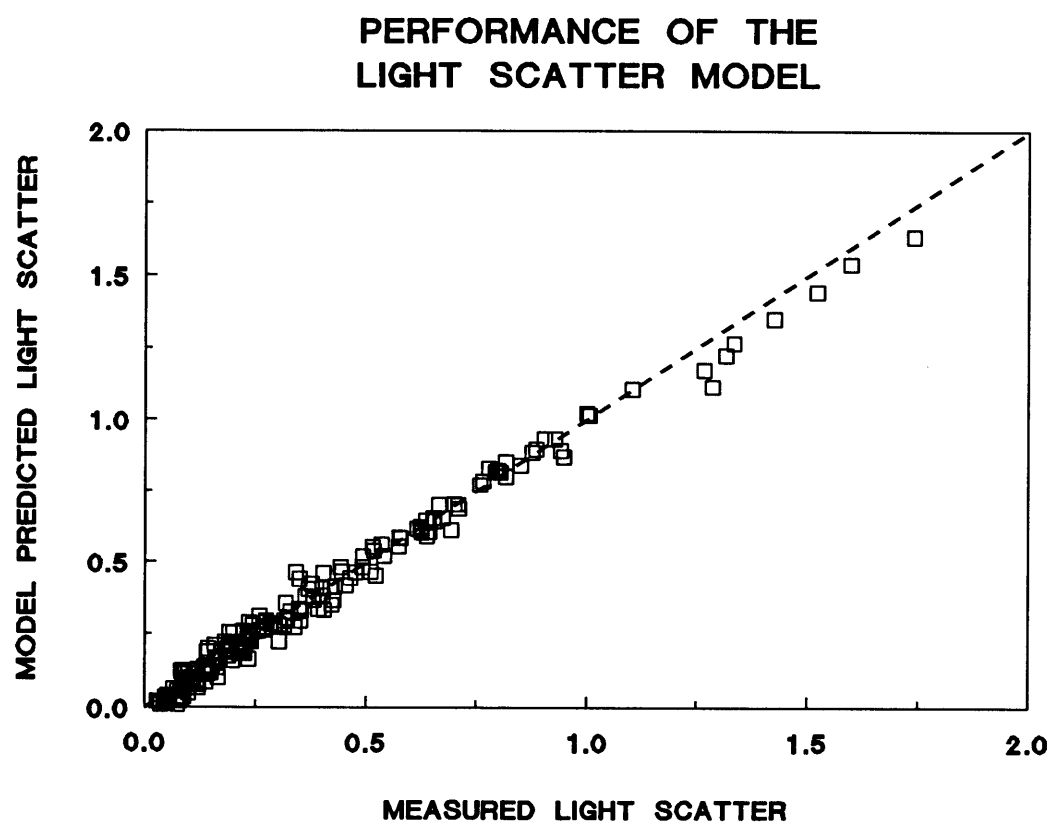


Figure 78: The goodness of fit of the model to the light scatter data.

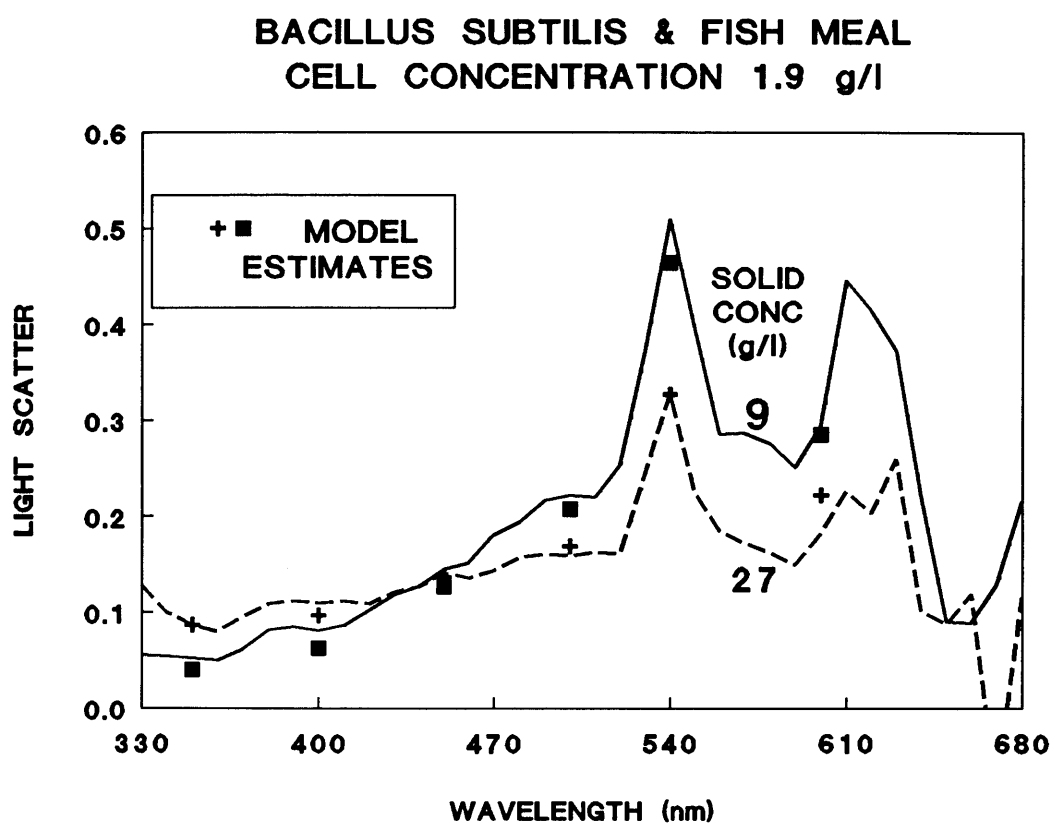


Figure 79: The use of the model to predict the light scatter spectrum. Data at low cell concentration.

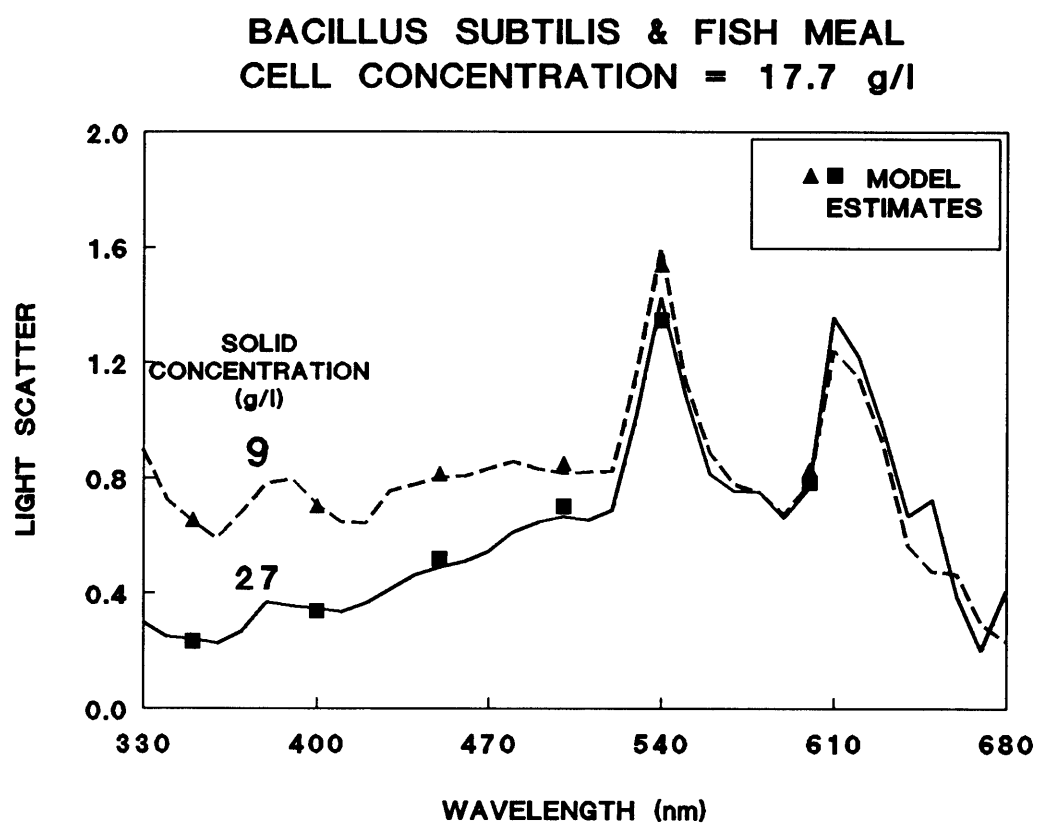


Figure 80: The use of the model to predict the light scatter spectrum. Data at high cell concentration.

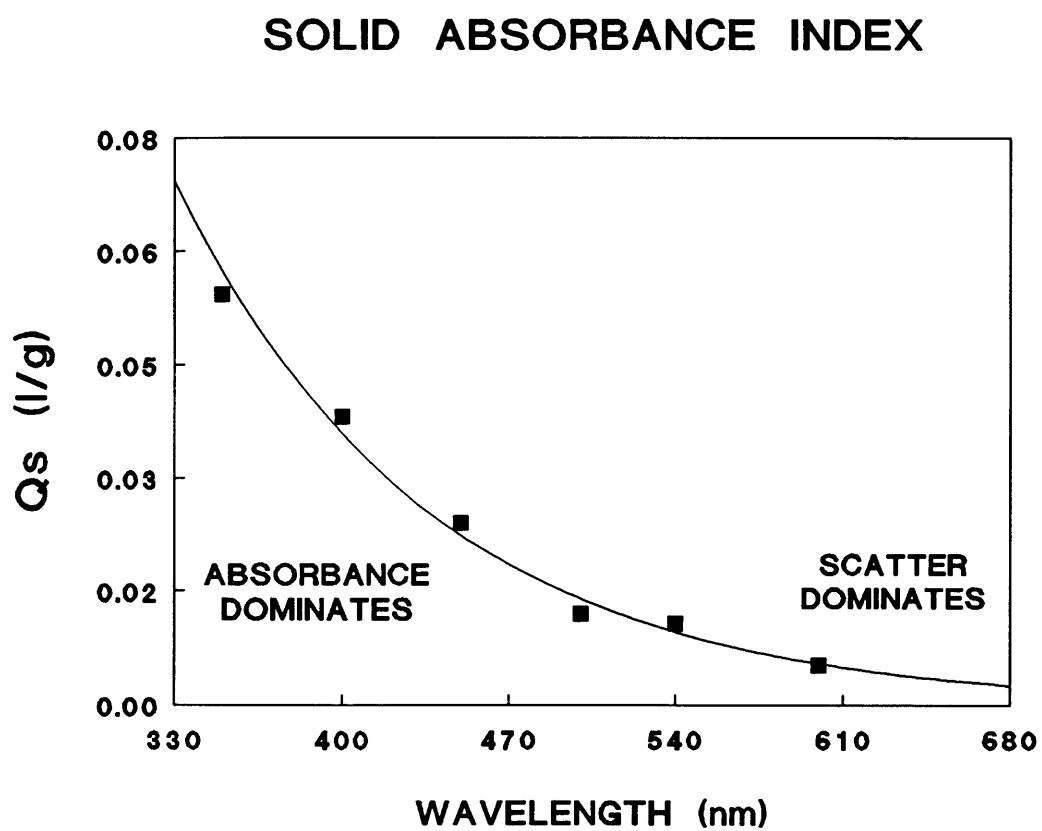


Figure 81: The solid absorbance index as a function of wavelength.

4.9) Evaluation of Fermentation Data.

A series of fermentations were conducted to evaluate the effect of fishmeal addition on protease specific and volumetric productivities. The measured profiles of the important variables, cell, fishmeal, glucose concentrations and alkaline protease activity from a batch fermentation can be seen in figure 82 to 85. The cell concentrations used in this analysis are the average of the light scatter and DNA technique results except in the overfeeding experiment where the carbon dioxide evolution technique was used. The carbon dioxide evolution technique was used because the light scatter technique was not available at the time of the fermentation and the large amounts of fishmeal added interfered with the DNA technique.

Protease formation is repressed by amino acids, see section 2.3.3. Thus, adding more fishmeal to a batch fermentation, can be expected to repress protease formation. Consistent with this, table 5 shows that when more fishmeal was used in a batch operation the protease specific and volumetric productivities decreased. Productivities were compared on the basis of time after the end of the lag phase. The lag phase was assumed to end when the carbon dioxide in the fermentor outlet gas reached 0.25%. Since glucose was not maintained the same in both the fermentations, the data in table 5 cannot be exclusively attributed to the effect of fishmeal. However, the data is consistent with the repression of protease formation by amino acids found in the literature.

The data in the batch fermentations was inconclusive because the glucose concentration was not the same in both fermentations. To investigate the effect of fishmeal addition, batch and fed-batch fermentations were conducted with similar glucose

concentration profiles. The glucose profiles of the two fermentations can be seen in figure 86. The glucose profiles were compared on the basis of time after the end of the lag phase. The data from these fermentations, see table 6, shows that the fed-batch fermentation increased the specific productivity by 2.5 times, and increased the volumetric productivity by 2.9 times.

Since more fishmeal was added in the fed-batch fermentation, the increase in productivities could have been due to the fact that more fishmeal was added, and not that it was added in small amounts during the fermentation. To test the hypothesis that it was not the total amount of fishmeal added that led to an increase in protease production, an overfeeding experiment was conducted. In the overfeeding fed-batch fermentation, approximately 3 times the amount of fishmeal was added than in the previous fed-batch fermentation. This large amount was added to show the effect of total fishmeal addition and also it was anticipated that by adding a large amount of fishmeal the effects caused by fluctuations in the glucose profile would be negligible in comparison.

The results from the fed-batch fermentations, see table 7, although they did not have identical glucose profiles, is consistent with the hypothesis that it is not the total amount of fishmeal added that led to the increase in protease production. It is unlikely that the 76% decrease in specific productivity was due to glucose fluctuations since the glucose concentrations in the overfeeding experiment were equal to or less than those in the base case for most of the fermentation, see figure 87. Since glucose was on average lower in the overfeeding experiment glucose's overall effect would have been to increase protease specific productivity. Thus the observed decrease in specific productivity can be attributed

to fishmeal addition.

To access the relative importance of glucose and fishmeal in controlling protease production a model was constructed. This model can be seen in appendix E. Although this model was not rigorous enough to support definite conclusions, it is hoped that it will act as a basis for further investigations in this area.

The literature shows that excess glucose and amino acids repress protease formation. The batch and fed-batch experiments show the importance of fishmeal addition on protease production. Consideration of these factors, indicates that a logical strategy for solid substrate addition could improve fermentation performance.

Table 5: The comparison of batch fishmeal fermentations.

	<u>MODE OF OPERATION</u>	
	BATCH	BATCH
Fishmeal Concentration Added Initially (g/l)	30	40
Alkaline Protease Activity (APU)	3.3	1.4
Cell Concentration (g/l)	10.4	14.4
Specific Productivity (APU.l/g-h)	0.030	0.010
Volumetric Productivity (APU/h)	0.31	0.14
Glucose Concentration Profiles	Not Similar	

Table 6: Comparison of a batch with a fed-batch fishmeal fermentation.

	<u>MODE OF OPERATION</u>	
	BATCH	FED-BATCH
Fishmeal Concentration Added Initially (g/l)	40	30
Fishmeal Concentration Added During the Fermentation (g/l)	0	68
Alkaline Protease Activity (APU)	1.4	4.6
Cell Concentration (g/l)	14.4	15.9
Specific Productivity (APU.l/g-h)	0.010	0.025
Volumetric Productivity (APU/h)	0.14	0.41
Glucose Concentration Profiles	Similar	

Table 7: The comparison of a base case with an overfeeding experiment in the fed-batch fermentation of fishmeal.

	<u>MODE OF OPERATION</u>	
	FED-BATCH BASE CASE	FED-BATCH OVER-FEEDING
Fishmeal Concentration Added Initially (g/l)	30	40
Fishmeal Concentration Added During the Fermentation (g/l)	68	260
Alkaline Protease Activity (APU)	4.6	4.3
Cell Concentration (g/l)	15.9	42.3
Specific Productivity (APU.l/g-h)	0.025	0.006
Volumetric Productivity (APU.l/g-h)	0.41	0.26
Glucose Concentration Profiles	Not similar	

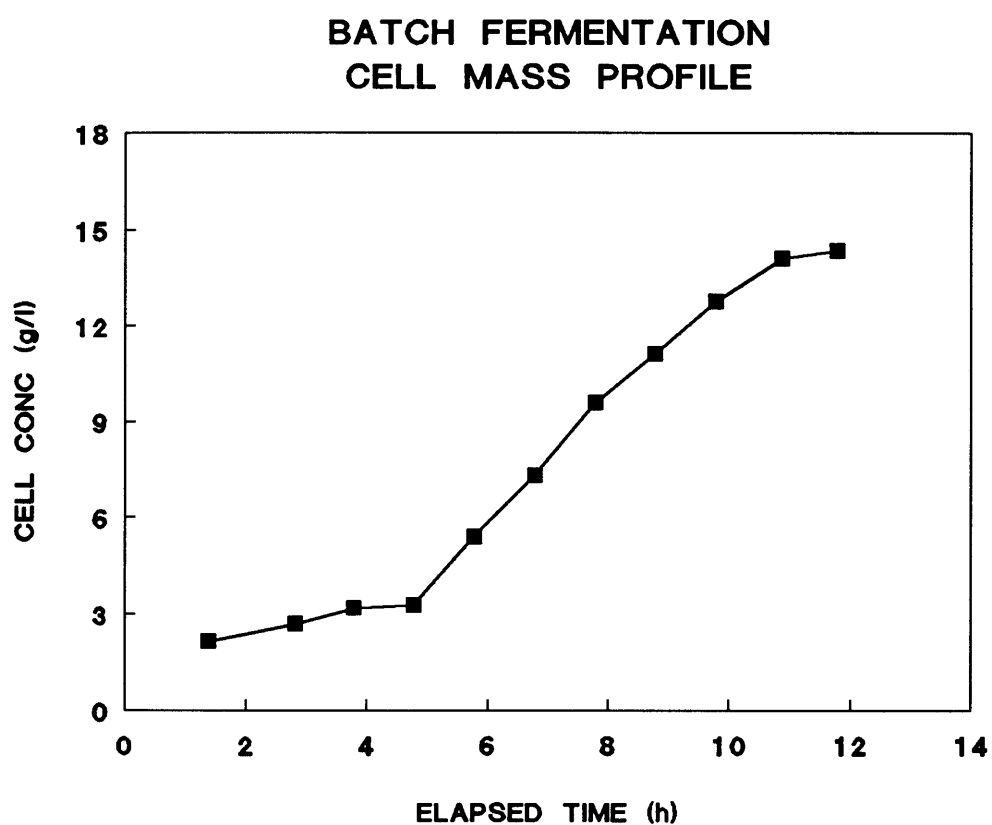


Figure 82: The cell concentration profile obtained during the batch fermentation of fishmeal by *Bacillus subtilis*.

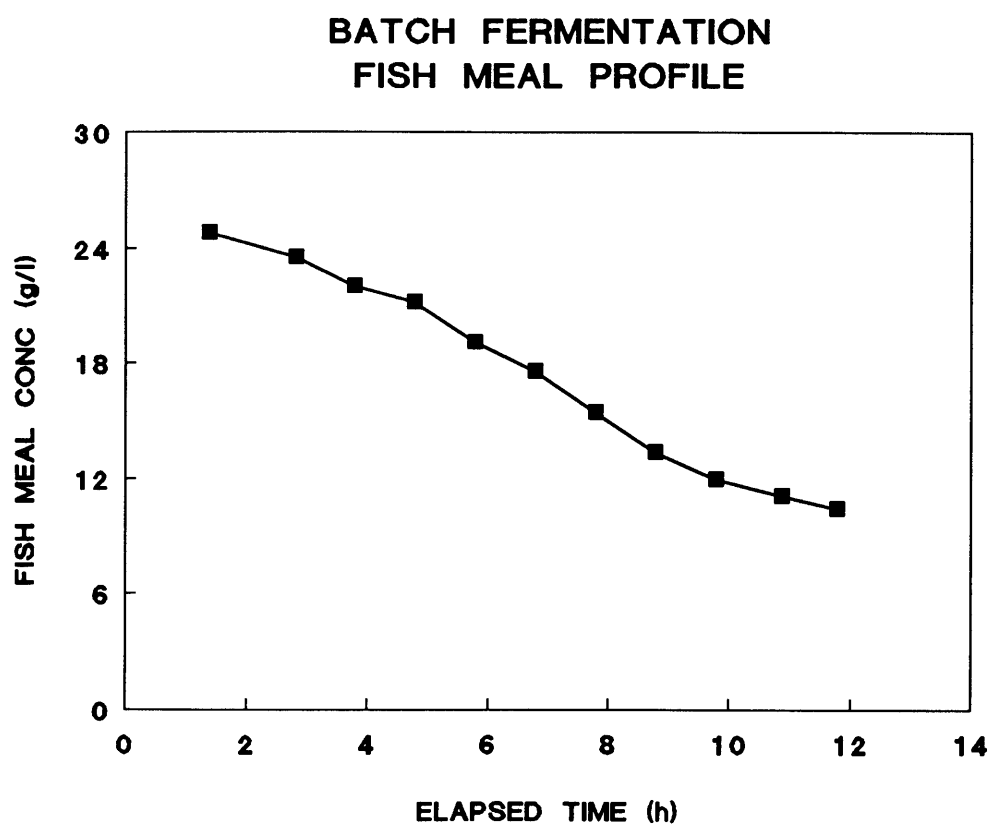


Figure 83: The fishmeal concentration profile obtained during the batch fermentation of fishmeal by *Bacillus subtilis*.

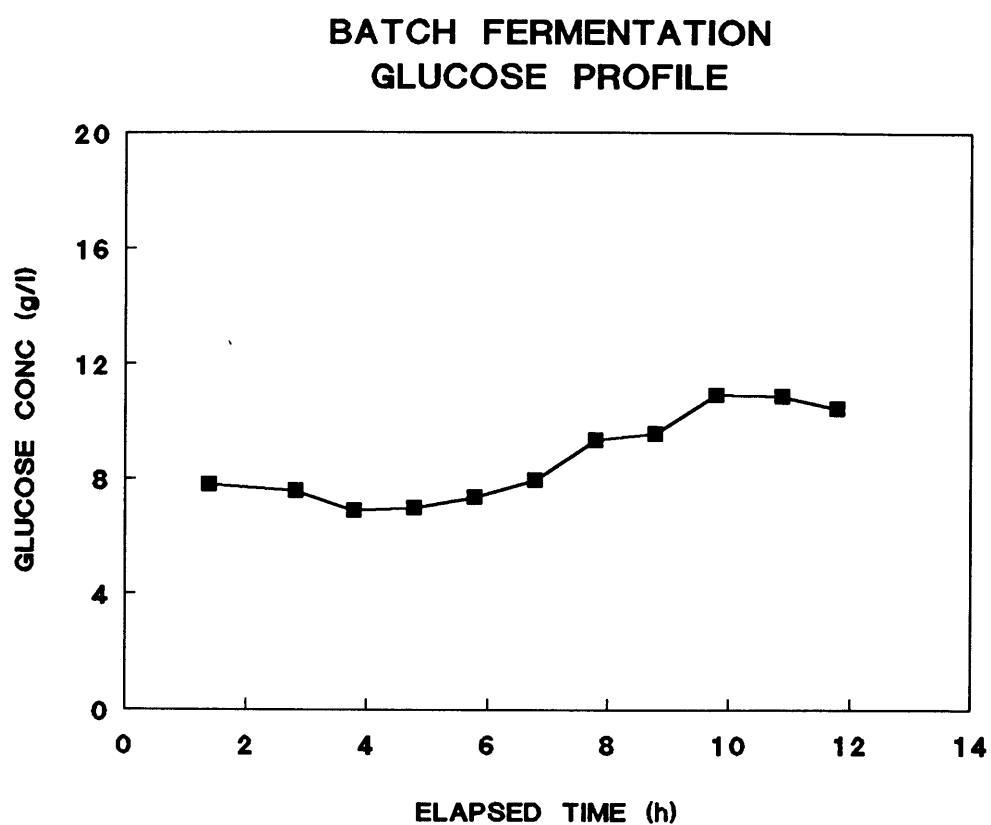


Figure 84: The glucose concentration profile obtained during the batch fermentation of fishmeal by *Bacillus subtilis*.

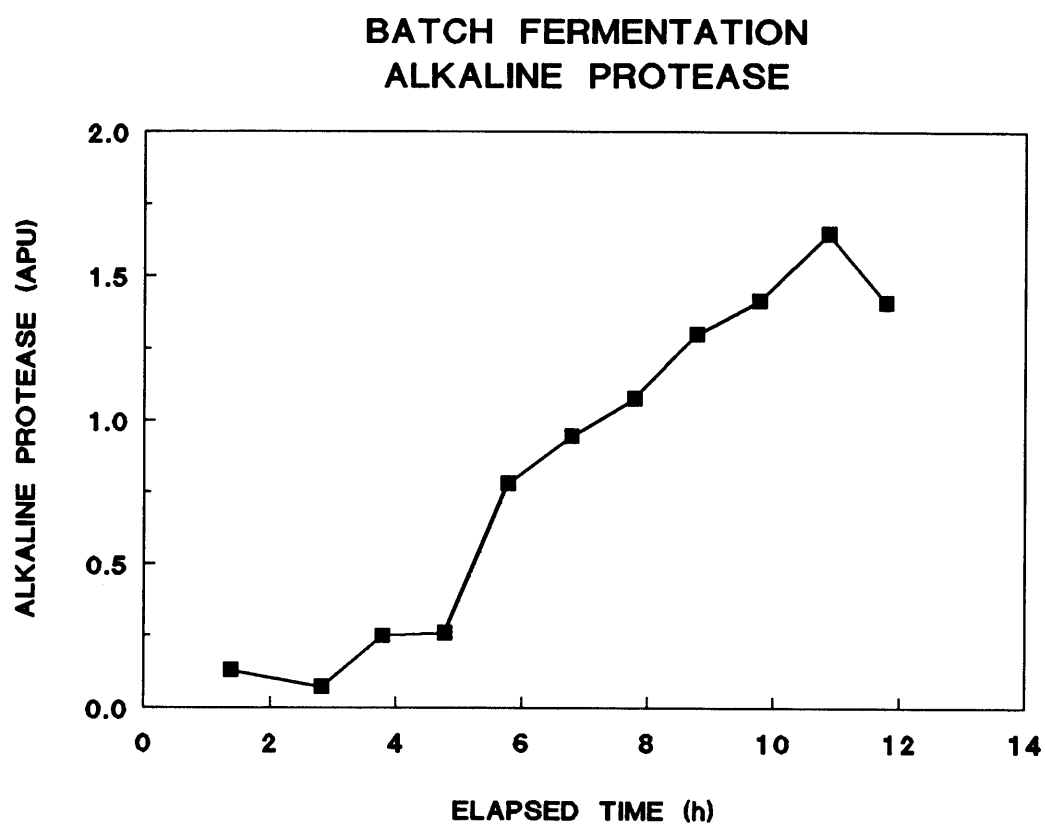


Figure 85: The alkaline protease activity profile obtained during the batch fermentation of fishmeal by *Bacillus subtilis*.

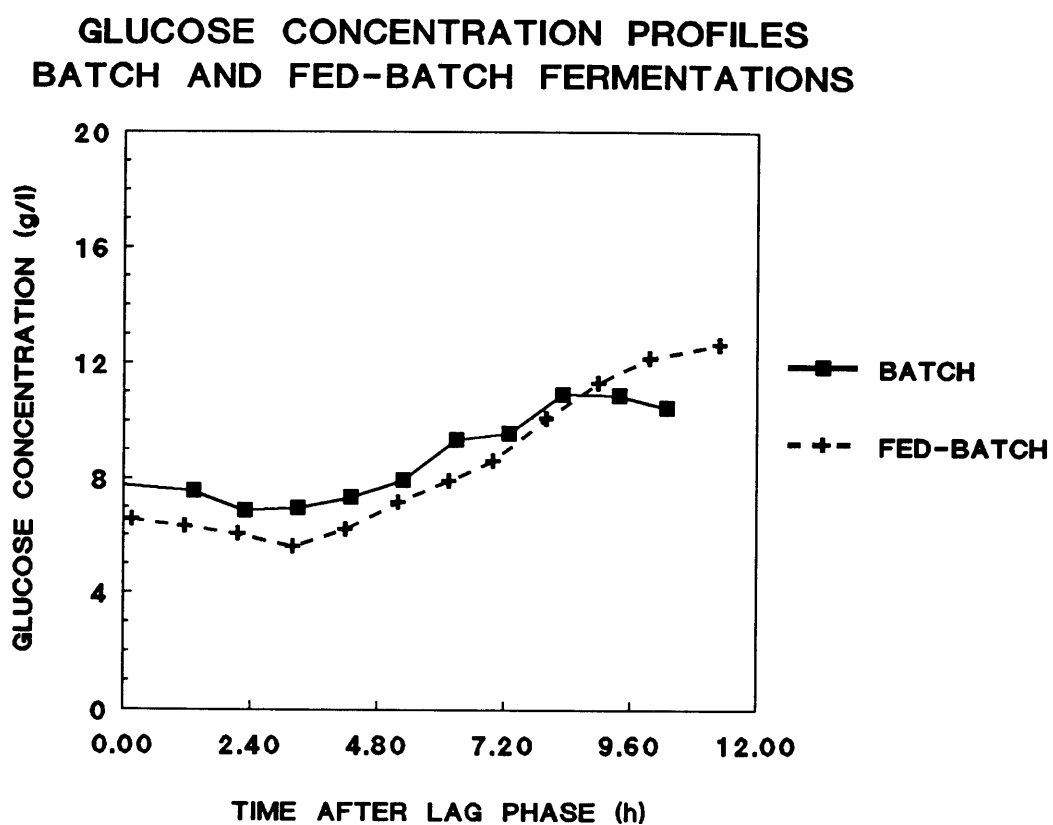


Figure 86: The glucose concentration profiles in the batch and fed-batch fermentations listed in table 6.

GLUCOSE CONCENTRATION PROFILES BASE CASE AND OVERFEEDING FERMENTATION

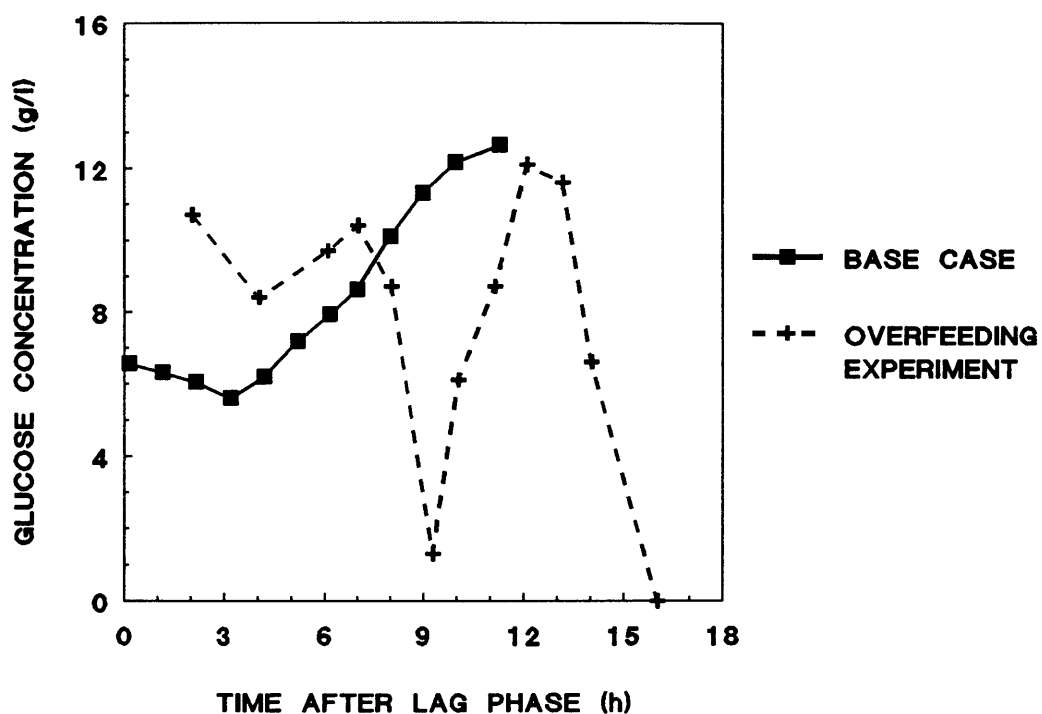


Figure 87: The glucose concentration profiles obtained in the fed-batch fermentations (base case and the overfeeding experiment) shown in table 7.

5) CONCLUSIONS

- 1) The light scattered by cells, measured using a front faced probe, is a function of cell concentration. This makes light scatter analysis a suitable cell concentration estimation technique.
- 2) The light scatter properties of cell and solid substrate mixtures were observed. The light scatter spectra of these mixtures exhibited "invariant regions". The invariant regions are regions where the solid substrate concentration does not affect the light scatter reading. Invariant regions were observed for a variety of cell/solid substrate systems.
- 3) Invariant regions were hypothesized to occur because the absorbance properties of the solid substrate change as a function of wavelength. At the invariant region it is hypothesized that the solid substrate scatters as much light as it absorbs. Hence, at the invariant region, a changing solid substrate concentration will not affect the light scatter reading.
- 4) Invariant regions have been used to estimate the cell concentration in the presence of solid substrate. The growth of *Bacillus subtilis* var *sakainensis* ATCC 21394 on fishmeal was used to test the technique.
- 5) The light scatter sensor was validated by comparing the technique to the DNA and carbon dioxide evolution techniques during a series of fermentations. The light scatter sensor was on average 0.9 g/l above the average of the carbon dioxide evolution and DNA techniques. The standard deviation of the difference between

the light scatter technique and the average of the carbon dioxide evolution and DNA techniques was 2.4 g/l.

- 6) A correction technique can be applied below 4.5 g/l to improve the accuracy of the light scatter technique in this region.
- 7) Batch and fed-batch fermentations, and a consideration of repression effects reported in the literature indicate that a reasoned control strategy for the addition of fishmeal to the fermentation is required.

6) SUGGESTIONS FOR FUTURE WORK

The results from this study have opened up other promising areas of research. Some suggestions and future challenges are as follows:

- 1) The application of the light scatter technique to other systems. The light scatter technique enabled cell concentration to be estimated in the presence of solid substrate. Other similar systems include the attachment of mammalian cells to microcarriers, immobilized cells and hollow fiber reactors. The question is, could the light scatter technique be adapted to work in such systems?
- 2) The effect of cellular morphology on the light scatter system. The uncertain effects of cellular morphology were deliberately avoided in this study. It remains to be seen whether the light scatter technique will work in systems of filamentous organisms, for example, those common in antibiotic production. The production of alkaline protease by *Fusarium* sp No. 5-128 would be an interesting model system to study. This would be a good model system because it produces protease and hence can grow on solid substrate. Most importantly this model system shows dimorphism between a mycelial form and a yeast form. In the early stage of the fermentation the microorganism develops as long, branched filaments. Later in the fermentation the microorganism is in the form of ovoid, yeast-like cells (Asai and Kono, 1984). Such a system would reveal the effects of changing morphology.

If the light scatter technique does turn out to be sensitive to cell morphology, this is not necessarily detrimental. Information on morphological changes is valuable in itself. Light scatter may be of use in following sporulation, inclusion body

formation or vacuole formation. With many recombinant products being manufactured, the ability to estimate inclusion body concentration on-line would be very valuable. In fact, light scatter has already been shown to increase dramatically when inclusion bodies are formed in *Escherichia coli* (Wittrup et al., 1988).

- 3) Performance Under Industrial Conditions. The probe described in this study is a laboratory instrument. The probe needs to be evaluated under industrial conditions. For example, the effect of more than one type of solid substrate or large amounts of oils in the fermentation medium, and the effect of gas bubbles, would need to be quantified if the probe were to be put into industrial usage. Also experiments need to be performed to see where the invariant region is located at high cell concentration (>20 g/l). This will enable the maximum cell concentration that the technique can measure to be determined.
- 4) Extension of the light scatter technique. The light scatter technique and the invariant region previously remained undiscovered because researchers assumed that the solid substrate would interfere with any light-based technique. The exact nature of this interference was not pursued.

Many other light scatter variables likewise remain untested. For example, light scatter has been demonstrated to be a function of the angle of the detector to the source (right angle versus front face geometry for example). The angular dependence of light scatter for solid substrate/cell mixtures could likely yield new information. It may be possible to estimate solid substrate concentration on-line by having two independent light scatter readings, one spectrum as a function of

wavelength and another as a function of angle. The ratio of light scatter readings at various angles could provide new information.

The application of mathematical deconvolution algorithms to separate the effects of cells and solid substrate from the spectrum may improve the accuracy of the light scatter technique.

- 5) Investigate other techniques. If cell/solid substrate mixtures exhibit invariant regions in the light scatter spectrum, what do the ultrasound scattering patterns of cell/solid substrate mixtures look like? Some work on the scatter of ultrasound in biological media has already been done (Javanaud, 1989) but no one has gone a step further to look at the ultrasound scattering of cells in solid substrate fermentations.

Another technique, used widely in molecular biology, is that of gene probes where a specific DNA sequence is identified. Some small DNA segments have the advantage of being extremely specific to a given organism. This technology is now well developed to identify bacteria (Barry et al., 1990). Thus it could potentially be used to distinguish between cells and solid substrate. The challenge in this area is to turn a well known laboratory technique into an on-line sensor.

- 6) Model Development. The model outlined in appendix E requires extensive additional work. Listed below are several of most important areas of further research.
 - a) Measurement of the Dissolved Fishmeal Concentration. The model is based on the effect of the dissolved fishmeal concentration. This dissolved fishmeal concentration was not validated. One potential validation tool would be to

measure the total nitrogen of cell and solid free fermentation samples. Since the dissolved fishmeal is the only source of nitrogen then the trend in total nitrogen should follow the trend in dissolved fishmeal.

- b) Alternate Growth Expressions. A Monod equation was used to describe the effect of dissolved fishmeal on growth. This model is known not to apply under non-steady state conditions. Alternate growth expressions need to be developed.
- c) Evaluation of Product Formation. The expression used to describe product formation assumes that product formation is related solely to growth. While such an expression is useful in fitting the data generated, it is in direct contradiction to literature observations that proteases are formed in the stationary phase. Thus a more rigorous expression taking into consideration stationary phase protease production is required.
- d) More Data Over a Wider Range. The model only considers data obtained over a very narrow range of the key variables, dissolved fishmeal and glucose. If the model is to be used for optimization and control strategy purposes then the effect of these variables needs to be considered over a wider range.
- e) Improvement of Glucose and Dissolved Fishmeal Profiles. The concentration of glucose and dissolved fishmeal are estimated based on a mass balance. While this approach is valid, a problem develops when the total concentration of a component going into or out of the aqueous phase is much greater than the concentration in the aqueous phase. Thus the concentration of, for

example glucose, in solution is determined as a small difference between two large numbers. A strategy should be devised to overcome this difficulty.

- f) Less Fitted Parameters. The model contains 17 parameters only two of which are independently determined. To turn the model from a curve fitting exercise into a something more meaningful either the parameters must be estimated independently or the total number of parameters decreased.
- g) Modeling of toxic product formation. The model suggests that toxic product formation led to a decrease in growth rate and eventually stopped maximum protease volumetric productivity being achieved. Modeling the production of toxic product formation, particularly acetate, and developing strategies to overcome the formation of toxic products could lead to increases in productivity. The presence of acetate and acetoin in the fermentation medium needs to be more rigorously confirmed experimentally.
- h) Developing Better Solid Substrate Feeding Strategies. The industrial production of protease involves the fed batch addition of solid substrate yet the feeding strategy for the solid substrate is viewed as so crucial that no details have been released in the open literature. Repression by amino acids plays such a large role in protease formation that the batch addition of large amounts of solid substrate is not rational whereas the controlled release of amino acids could avoid repression. Far less attention has been placed on solid substrate feeding than the feeding of glucose to fermentations. The development of successful solid substrate feeding strategies based on rational

design, as opposed to an empirical approach, would be of considerable significance.

7) REFERENCES

Aber Instruments Ltd., Aberystwyth Science Park, Aberystwyth, SY23 3AH, United Kingdom.

Accurate Chemical and Scientific Corporation, 300 Shames Drive, Westbury, NY 11590. Telephone (516)443-4900. Catalog # 18860, Data sheet, "**DAPI-The New Fluorescent DNA Stain.**", received 1988.

Adler-Nissen, J. "**Enzymic Hydrolysis of Food Proteins**", Elsevier Applied Science Publishers, London, 1986.

Agar, D.W. "**Microbial Growth Rate Measurement Techniques**", Chapter 17 in volume 4 of *Comprehensive Biotechnology*, edited by M. Moo-Young, Pergamon Press, New York, 1985.

Agudu, E.W. "**The Effect of Fishmeal or Fermacto 500 Supplementation on the Performance of Laying Hens**", *Ghana Journal of Agricultural Science*, 4, 211-214, 1971.

Aidoo, K.E., Hendry, R. and Wood, B.J.B. "**Estimation of Fungal growth in a Solid State Fermentation**", *European Journal of Applied Microbiology and Biotechnology*, 12, 6-9, 1981.

Allison, C. and MacFarlane, G.T. "**Protease Production by *Clostridium perfringens* in Batch and Continuous Culture**", *Letters in Applied Microbiology*, 9, 45-48, 1989.

Allwood, M.C. and Russell, A.D. "**Thermally Induced Changes in the Physical Properties of *Staphylococcus aureus***", *Journal of Applied Bacteriology*, 32, 68-78, 1969.

Amin, P.M., Nigam, J.N., Lonsane, B.K., Baruah, B., Singh, H.D., Baruah, J.N. and Iyengar, M.S. "**Microbial Biomass Production on Solid Hydrocarbon**", *Fiolia Microbiol.*, 18, 49-55, 1973.

Anson, M. L. "**The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin**", *Journal of General Physiology*, 22(1), 79-89, 1938.

Apel, W.A., Dugan, P.R., Filppi, J.A. and Rheins, M.S. "**Detection of *Thiobacillus ferrooxidans* in Acid Mine Environments by Indirect Fluorescent Antibody Staining**", *Applied and Environmental Microbiology*, 32(1), 159-165, 1976.

Archer, M.C., Ragnarsson, J.O., Tannenbaum, S.R. and Wang, D.I.C. "**Enzymatic Solubilization of an Insoluble Substrate, Fish Protein Concentrate: Process and Kinetic Considerations**", *Biotechnology and Bioengineering*, 15, 181-196, 1973.

Arnesen, G. "**Total and Free Amino Acids in Fishmeals and Vacuum-Dried Codfish Organs, Flesh, Bones, Skin and Stomach Contents**", *Journal Sci. Fd. Agric.*, 20, 218-220, 1969.

Asai, T. and Kono, T. **"Industrial Application of Fermentation Kinetics"**, Advances in Biotechnological Processes, Volume 3, Edited by A. Mizrahi and A.L. van Wezel, 53-79, Alan R. Liss Inc., New York, 1984.

Aunstrup, K. **"Industrial Production of Proteolytic Enzymes"**, Industrial Aspects of Biochemistry, Volume 30, Part 1, Edited by B. Spenser, North Holland/American Elsevier, 1974.

Aunstrup, K. **"Proteinases"**, Chapter 2, Microbial Enzymes and Bioconversions, Economic Microbiology, Volume 5, Edited by A.H. Rose, Academic Press, New York, 1980.

Aunstrup, K., Andersen, O. and Outtrup, H. **"Proteolytic Enzymes, their Production and Use"**, US Patent, 3,723,250; 1973

Aunstrup, K. and Outtrup, H. **"Preparation of Proteolytic Enzyme Preparations"**, British Patent, 1,205,403; 1970.

Aunstrup, K. and Outtrup, H. **"Improvements in or relating to the Preparation of Microbial Enzymes"**, British Patent, 1,303,633; 1973.

Bailey, J.E. and Ollis, D.F. **"Biochemical Engineering Fundamentals"**, Second Edition, McGraw-Hill Book Company, New York, 1986.

Barfoed, H.C. **"Detergents"**, Chapter 4.7 in Industrial Enzymology: The Application of Enzymes to Industry, Edited by T. Godfrey and J. Reichelt, The Nature Press, New York, 1983.

Barry, T., Powell, R. and Gannon, F. **"A General Method to Generate DNA Probes for Microorganisms"**, Bio/Technology, 8, 233-236, 1990.

Baserga, R. **"Measuring Parameters of Growth"**, Chapter 1 in Cell Growth and Division - A Practical Approach edited by R. Baserga, IRL Press, Oxford, 1989.

Bates and Bower, **"Alkaline Solutions for pH Control"**, Analytical Chemistry, 28(8), 1322-1324, 1956.

Bayer, K. and Fuehrer, F. **"Computer Coupled Calorimetry in Fermentation"**, Process Biochemistry, July/August, 1982.

Beermann, D.H. and Hogue, D.E. **"Effect of Cimaterol and Fishmeal on Body Composition and Skeletal Muscle Growth in Lambs"**, Proceedings of the Cornell Nutrition Conference for Feed Manufacturers, 29-38, 1986.

Benson, M. C., McDougal, D.C. and Coffey, D.S. **"The Application of Perpendicular and**

Forward Light Scatter to Assess Nuclear and Cellular Morphology", Cytometry, 5, 515-522, 1984.

Berkman, R.M. and Wyatt, P.J. **"Differential Light Scattering Measurements of Heat-Treated Bacteria"**, Applied Microbiology, 20(3), 510-512, 1970.

Boidin, A. and Effront, J. **"Process of Manufacturing Diastase and Toxins by Oxidizing Ferments"**, US Patent, 1,227,525; 1917.

Boquien, C.Y., Corrieu, G. and Desmazeaud, M.J. **"Enzymatic Methods for Determining Populations of *Streptococcus cremoris* AM2 and *Leuconostoc lactis* CNRZ 1091 in Pure and Mixed Culture"**, Applied Microbiology and Biotechnology, 30, 402-407, 1989.

Brown, H.C. **"A Preliminary Note on Experimental Researches Connected with the Standardisation of Vaccines"**, The Indian Journal of Medical Research, 1, 711-725, 1914.

Brown, H.C. **"Further Observations on the Standardization of Bacterial Suspensions"**, The Indian Journal of Medical Research, 7, 238-250, 1919.

Brown, R.G.W. **"Laser Fibre Optics in Biotechnology"**, Trends in Biotechnology, 3(8), 200-203, 1985.

Brown, H.G. and Kirwan, E.W. **"Standardization of Bacterial Suspensions by Opacity"**, The Indian Journal of Medical Research, 2, 763-769, 1914.

Brunk, C. F. and James, T. W. **"Intracellular and Invitro Fluorescence of the DNA Specific Probe : 4'6-Diamidino-2-phenyl-indole (DAPI)."**, Journal of Cell Biology, Abstracts of the American Society for Cell Biology, 75(2), CR618, p136, 17th Annual Meeting, 15-18 November, San Diego, CA., 1977.

Brunk, C.F., Jones, K.C. and James, T.W., **"Assay for Nanogram Quantities of DNA in Cellular Homogenates"**, Analytical Biochemistry, 92, 497-500, 1979.

Brunsting, A. and Mullaney, P.F. **"Light Scattering from Coated Spheres: Model for Biological Cells"**, Applied Optics, 11(3), 1972.

Bulla, L.A. and Hoch, J.A. **"Biology of the *Bacilli*"**, Chapter 3, Biology of Industrial Microorganisms, Edited by A.L. Demain and N.A. Solomon, The Benjamin/Cummings Publishing Company Inc., London, 1985.

Carleysmith, S.W. and Fox, R.I. **"Fermenter Instrumentation and Control"**, Advances in Biotechnological Processes 3, Edited by A. Mizrahi and A.L. van Wezel, p 1-51, Alan R. Liss., 1984.

Carr, R. **"Applications of Dynamic Light Scattering to Biotechnology"**, World Biotech Report, 381-388, Online Publications, London, 1988.

Carrizalez, V., Rodriguez, H. and Sardina, I. **"Determination of the Specific Growth of Molds on Semi-Solid Cultures"**, Biotechnology and Bioengineering, 23, 321-333, 1981.

Cejka, A. **"Preparation of Media"**, Chapter 26, Biotechnology, Volume 2, Edited by H Brauer, VCH, Weinheim, 1985.

Chaloupka, J. and Kreckova, P. **"Regulation of the Formation of Protease in *Bacillus megaterium*: I The Influence of Amino Acids on the Enzyme Formation"**, Folia Microbiologica, 11, 82-88, 1966a.

Chaloupka, J. and Kreckova, P. **"Regulation of the Formation of Protease in *Bacillus megaterium*: II Formation of the Enzyme by Non-growing Cells"**, Folia Microbiologica, 11, 89-94, 1966b.

Charney, J. and Tomarelli, R. M. **"A Colorimetric Method for the Determination of the Proteolytic Activity of Duodenal Juice"**, The Journal of Biological Chemistry, 171, 501-505, 1947.

Cheftel, C., Ahern, M., Wang, D.I.C. and Tannenbaum, S.R. **"Enzymatic Solubilization of Fish Protein Concentrate: Batch Studies Applicable to Continuous Enzyme Recycling Processes"**, J. Agr. Food Chem., 19(1), 155-161, 1971.

Churchill, B.W. and Buss, D.R. **"Composition of Matter and Process"**, US patent 3,740,318; 1973.

Clarke, D.J., Blake-Colman, B.C., Carr, R.J., Calder M.R. and Atkinson, T. **"Monitoring Reactor Biomass"**, Trends in Biotechnology, 4(7), 173-178, 1986.

Collins, C.H. and Lyne, P.M. **"The Gram-Positive Spore Bearers: *Bacillus* and *Clostridium*"**, Chapter 28 in Microbiological Methods, Butterworths, Boston, 1976.

Coombs, J. **"Dictionary of Biotechnology"**, Elsevier Science Publishing Company Inc., New York, 1986.

Cooney, C.L. **"Growth of Microorganisms"**, Chapter 2 in volume 1 of Biotechnology, edited by H.-J. Rehm and G. Reed, VCH, Weinheim, 1981.

CRC Handbook of Microbiology, Volume 3, **"Microbial Composition: Amino Acids, Proteins, and Nucleic Acids"**, Edited by A.I. Laskin and H.A. Lechevalier, 2nd Edition, CRC Press, Boca Raton, Florida, p855, 1981.

Damiano, D. and Wang, S.S. "Improvements in Ethanol Concentration and Fermentor Ethanol Productivity in Yeast Fermentations using Whole Soy Flour in Batch, and Continuous Recycle Systems", *Biotechnology Letters*, 7(2), 135-140, 1985.

Dean, D.H. and Zeigler, D.R. "*Bacillus* Genetic Stock Center Strains and Data", Fourth Edition, The Ohio State University, Department of Biochemistry, 484 West 12th Avenue, Columbus, Ohio 43210.

Debabov, V.G. "*The Industrial Use of Bacilli*", *The Molecular Biology of the Bacilli*, Volume I, *Bacillus subtilis*, Edited by D.A. Dubnau, Academic Press, 1982.

Dekovich, T., Mandel, W., Hennigan, P., Tsai, L.B. and Fenton, D. "Direct Photometric Measurement of *E. Coli* Cell Density in Fermentors", American Society for Microbiology Meeting, New Orleans, Louisiana, May 18, 1989.

Delgado, G., Topete, M. and Galindo, E. "Interaction of Cultural Conditions and End-Product Distribution in *Bacillus subtilis* Grown in Shake Flasks", *Applied Microbiology and Biotechnology*, 31, 288-292, 1989.

Difco Laboratories, "Difco Manual, Dehydrated Culture Media and Reagents for Microbiology", Tenth Edition, Detroit, 1984.

Egorov, N.S. "*Antibiotics a Scientific Approach*", Translated from the Russian by A. Rosinkin, MIR Publishers, Moscow, 1985.

Espejo, R.T. and Ruiz, P. "Growth of Free and Attached *Thiobacillus ferrooxidans* in Ore Suspension", *Biotechnology and Bioengineering*, 30, 586-592, 1987.

Feldman, L.I. "Preparation of Microbial Alkaline Protease by Fermentation with *Bacillus subtilis* variety *Licheniformis*", US patent, 3,623,957; 1971.

Findlay, R.H., King, G.M. and Watling, L. "Efficacy of Phospholipid Analysis in Determining Microbial Biomass in Sediments", *Applied and Environmental Microbiology*, 55(11), 2888-2893, 1989.

Fogarty, W.M. and Kelly, C.T. "Amylases, Amyloglucosidases and Related Glucanases", Chapter 3, *Microbial Enzymes and Bioconversions*, *Economic Microbiology*, Volume 5, Edited by A.H. Rose, Academic Press, New York, 1980.

Fukumoto, J., Yamamoto, T. and Tsuru, D. "Process for Producing Detergent Resisting Alkaline Protease", US Patent, 3,838,009; 1974.

Fukushima, Y., Itoh, H., Fukase, T. and Hiroshi, M. "Continuous Protease Production in a Carbon-Limited Chemostat Culture by Salt Tolerant *Aspergillus oryzae*", *Applied*

Microbiology and Biotechnology, 30, 604-608, 1989.

Gerrard, H.N., Parker, M.S. and Porter, G.S. **"The Effect of Pipetting on the Concentration of Homogeneous Spore Suspensions"**, Journal of Pharmacy and Pharmacology, 13, 405-411, 1961.

Ghaly, A.E., Kok, R. and Ingrahm, J.M. **"Growth Rate Determination of Heterogeneous Microbial Population in Swine Manure"**, Applied Biochemistry and Biotechnology, 22, 59-78, 1989.

Ghosh, S., Pohland, F.G. and Gates, W.E. **"Phasic Utilization of Substrates by Aerobic Cultures"**, Journal of the Water Pollution Control Federation, 44(3), 376-400, 1972.

Gomori, **"Preparation of Buffers for Use in Enzyme Studies"**, Methods in Enzymology, 1, 138-146, 1955.

Gordon, R.E. **"The Genus *Bacillus*"**, CRC Handbook of Microbiology, 2nd Edition, Volume 1, Bacteria, Edited by Laskin, A.I. and Lechevalier, H.A., CRC Press Inc., 1977.

Greene, R.V. and Gordon, S.H. **"Monitoring Solid-Substrate Fermentations by Fourier Transform Infrared-Photoacoustic Spectroscopy"**, Presented at PACIFICHEM '89 conference in Hawaii, 1989.

Grishin, S.I. and Tuovinen, O.H. **"Scanning Electron Microscope Examination of *Thiobacillus ferrooxidans* on Different Support Matrix Materials in Packed Bed and Fluidized Bed Bioreactors"**, Applied Microbiology and Biotechnology, 31, 505-511, 1989.

Güntelberg, A.V. **"A Method for the Production of the Plakalbumin-Forming Proteinase from *Bacillus Subtilis*"**, Comptes Rendus des travaux du Laboratoire Carlsberg, Série Chimique, 29(3), 27-35, 1954.

Gusek, T.W., Wilson, D.B. and Kinsella, J.E. **"Influence of Carbon Source on Production of a Heat Stable Protease from *Thermomonospora fusca* YX"**, Applied Microbiology and Biotechnology, 28, 80-84, 1988.

Hafiz, A.H. and Qadeer, M.A. **"Biosynthesis of Proteases by Submerged Culture Fermentation"**, Pakistan Journal Sci. Ind. Res., 29(2), 108-110, 1986.

Hagihara, B. **"Bacterial and Mold Proteases"**, Chapter 9, Volume 4, The Enzymes, Edited by P.D. Boyer, H. Lardy and K. Myrback, Second Edition, 193-214, 1960.

Han, Y.W. **"Oxygen Requirement for Growth of *Candida utilis* on Semisolid Straw Substrate"**, Biotechnology and Bioengineering, 30, 672-674, 1987.

Hancher, C.W., Thacker, L.H. and Phares, E.F. **"A Fiber-Optic Reflective Turbidimeter for Continuously monitoring Cell Concentration during Fermentation"**, Biotechnology and Bioengineering, 16, 475-484, 1974.

Harris, C.M. and Kell, D.B. **"The Estimation of Microbial Biomass"**, Biosensors, 1, 17-84, 1985.

Harris, C.M., Todd, R.W., Bungard, S.J., Lovitt, R.W., Morris, G. and Kell, D.B. **" Dielectric Permittivity of Microbial Suspensions at Radio Frequencies: A Novel Method for the Real-Time Estimation of Microbial Biomass"**, Enzyme and Microbial Technology, 9, 181-186, 1987.

Hashimoto S., Fujita, M. and Baccay, R.A. **"Biomass Determination in the Anaerobic Digestion of Night Soil"**, Journal of Fermentation Technology, 60(1), 51-54, 1982.

Heineken, F.G. and O'Connor, R.J. **"Continuous Culture Studies on Biosynthesis of Alkaline Protease, Neutral Protease and α -Amylase by *Bacillus subtilis* NRRL-B3411"**, Journal of General Microbiology, 73, 35-44, 1972.

Hevia, P. and Olcott, H.S. **"Flavor of Enzyme-Solubilized Fish Protein Concentrate Fractions"**, Journal Agric. Food Chem., 25(4), 772-775, 1977.

Hevia, P., Whitaker, J.R. and Olcott, H.S. **"Solubilization of a Fish Protein concentrate with Proteolytic Enzymes"**, J. Agric. Food Chem., 24(2), 283-285, 1976.

Hjortkjaer, P., Novo Nordisk A/S, Novo Allé, 2880 Bagsvaerd, Denmark, private communications, 1990.

Hong, K., Tanner, R.D., Malaney, G.W. and Wilson, D.J. **"A Spectrophotometric Method for Estimating the Yeast Cell Concentration in a Semi-Solid State Fermentation"**, Process Biochemistry, 22, 5, 149-153, 1987.

Hsu, J.-P. **"Bacterial Adhesion and Digestion of a Particulate Substrate"**, Presented at the American Chemical Society, 194th National Meeting, Microbial and Biochemical Technology Division, August 30-September 4, New Orleans, LA, sent to J. Theor. Biol., 1987.

Huang, T.-L., Han, Y.W. and Callihan, C.D. **"Application of the Lowry Method for Determination of cell Concentration in Fermentation of Waste Cellulosics"**, Journal of Fermentation Technology, 49(6), 574-576, 1971.

Imada, y. and Takahashi, K. **"Process for Producing Steroidal Alcohols"**, US Patent, 4,223,091; 1980.

Imming, G., Schaller, K. and Meiners, M. **"The Use of Automated Analysers to Control**

Microbial Processes", p27-32, in **Modelling and Control Of Biotechnical Processes**, Proceedings of the First International Federation of Automatic Control Workshop, Helsinki, Finland, Edited by A Halme, Pergammon Press, New York, 1982.

Ito, K., Kimizuka, A., Okazaki, N. and Kobayashi, S. **"Mycelial Distribution in Rice Koji"**, Journal of Fermentation and Bioengineering, 68(1), 7-13, 1989.

Jacobsen, F. **"Effect of Enzymatic Treatment of Stickwater on Evaporator Capacity and Fouling"**, Process Biochemistry, 103-108, August, 1985.

Jacobsen, F. and Lykke-Rasmussen, O. **"Energy Savings Through Enzymatic Treatment of Stickwater in the Fishmeal Industry"**, Process Biochemistry, 165-169, October, 1984.

Javanaud, C. **"The Application of a Fractal Model to the Scattering of Ultrasound in Biological Media"**, Journal of the Acoustical Society of America, 86(2), 493-496, 1989.

Joglekar, R., Clerman, R., Ouellette, R. and Cheremisinoff, P. **"Biotechnology in Industry - Selected Applications and Unit Operations"**, Ann Arbor Science, 1983.

Joep, C., James, T. and Wildman, S. **"Visualization of Higher Plant Chloroplast DNA by Fluorescence Microscopy with 4',6-Diamidino-2-phenylindole (DAPI)."**, Journal of Cell Biology, Abstracts of the American Society for Cell Biology, 75(2), MC413, p304, 17th Annual Meeting, 15-18 November, San Diego, CA., 1977.

Ju, N.-H., Damiano, D., Shin, C.-S., Kim, N.-K. and Wang, S.S. **"Continuous Ethanol Fermentation of *Zymomonas mobilis* using Soy Flour as a Protective Agent"**, Biotechnology Letters, 5(12), 837-842, 1983.

Junker, B.H. **"Monitoring and Assessment of Aqueous/Perfluorocarbon Fermentation Systems"**, PhD Thesis, Massachusetts Institute of Technology, 1988.

Junker, B.H., Wang, D.I.C. and Hatton, T.A. **"Fluorescence Sensing of Fermentation Parameters Using Fiber Optics"**, Biotechnology and Bioengineering, 32, 55-63, 1988.

Kalabokias, G. **"Preparation of Microbial Alkaline Protease by Fermentation with *Bacillus subtilis* variety Licheniformis"**, US patent 3,623,956; 1971.

Kalisz, H.M. **"Microbial Proteinases"**, Advances in Biochemical Engineering/Biotechnology, Volume 36, 'Enzyme Studies', Edited by A. Fiechter, 1-65, Springer-Verlag, New York, 1988.

Kapuscinski, J. and Skoczylas, B. **"Simple and Rapid Fluorimetric Method for DNA Microassay."**, Analytical Biochemistry, 83, 252-257, 1977.

Karube, I. **"Biosensors in Fermentation and Environmental Control"**, Chapter 11 in

Biotechnology - Applications and Research, edited by P. Cheremisinoff and Ouellette, Technomic Publishing Co. Inc., Basel, 1985.

Keay, L. **"Microbial Proteases"**, Process Biochemistry, 17-21, August, 1971.

Keay, L., Moseley, M.H., Anderson, R.G., O'Connor, R.J. and Wildi, B.S. **"Production and Isolation of Microbial Proteases"**, Biotechnology and Bioengineering Symposium No. 3, 63-92, 1972.

Khandeparkar, V.G. **"Release of Gossypol from Cottonseed Meal in Fermentation Medium"**, Indian Journal of Experimental Biology, 19, 574-576, 1981.

Kleyn, J.G. and Vacano, N.L. **"Some Effects of Soybeans on Yeast Growth and Beer Flavor"**, The Brewers Digest, 41(11), 95-99, 1966.

Klimov, N.A., Batarin, V.I., Rybal'chenko, O.V. and Andreev, O.A. **"Formation of Extracellular Protease by Cells of Thermophilic Bacteria"**, Translated from Mikrobiologiya, 57(4), 579-585, 1988.

Koch, A.L. **"Some Calculations on the Turbidity of Mitochondria and Bacteria"**, Biochimica and Biophysica Acta, 51, 429-441, 1961.

Kole, M.M., Draper, I. and Gerson, D.F. **"Protease Production by *Bacillus subtilis* in Oxygen-Controlled, Glucose Fed-Batch Fermentations"**, Applied Microbiology and Biotechnology, 28, 404-408, 1988a.

Kole, M.M., Draper, I. and Gerson, D.F. **"Protease Production by *Bacillus subtilis* Using Simultaneous Control of Glucose and Ammonium Concentrations"**, Journal of Chemical Technology and Biotechnology, 41, 197-206, 1988b.

Kotlarchyk, M. **"Light Scattering from Cells"**, Masters Thesis, Massachusetts Institute of Technology, Cambridge, 1978.

Küenzi, M.T. **"Process Design and Control in Antibiotic Fermentations"**, Antibiotics and Other Secondary Metabolites, Biosynthesis and Production, Federation of European Microbiological Societies Symposium No. 5, Edited by R Hütter, T Leisinger, J. Nüesch and W. Wehrli, 39-56, Academic Press, New York, 1978.

Kunitz, M. **"Crystalline Soybean Trypsin Inhibitor, 2: General Properties"**, Journal of General Physiology, 30(4), 291-310, 1947.

Kurane, R., Suzuki, T., Takahara, Y., Kurita, N. and Miyaji, M. **"Application of Fluorescent Antibody Staining Technique to Trace the Microorganism Inoculated in Biological treatment Systems"**, Agricultural and Biological Chemistry, 43(10), 2093-2098, 1979.

- Layman, P.L. **"Industrial Enzymes Battling to Remain Specialties"**, Chemical and Engineering News, 64(37), 11-14, 1986.
- Lee, Y.H. **"Pulsed Light Probe for Cell Density Measurement"**, Biotechnology and Bioengineering, 23, 1903-1906, 1981.
- Leifson, E. **"Atlas of Bacterial Flagellation"**, Academic Press, New York, 1960.
- Leung, J. C-Y. **"Production of Acetone and Butanol by *Clostridium acetobutylicum* Using Free and Immobilized Cells"**, PhD thesis, Massachusetts Institute of Technology, 1982.
- Leusch, H.G., Hoffmann, M. and Emeis, C.C. **"Fluorometric Determination of the Total DNA Content of Different Yeast Species using 4',6-diamidino-2-phenyl-indol-dihydrochloride"**, Canadian Journal of Microbiology, 31(12), 1164-1166, 1985.
- Levisohn, S. and Aronson, A.I. **"Regulation of Extracellular Protease Production in *Bacillus cerus*"**, Journal of Bacteriology, 93(3), 1023-1030, 1967.
- Liebs, P., Riedel, K., Graba, J.P., Schrapel, D. and Tischler, U. **"Formation of Some Extracellular Enzymes During the Exponential growth of *Bacillus subtilis*"**, Folia Microbiol., 33, 88-96, 1988.
- Linke, H.A.B., Mechlinski, W. and Schaffner, C.P. **"Production of Amphotericin B-¹⁴C by *Streptomyces nodosus* Fermentation, and Preparation of the Amphotericin B-¹⁴C-Methyl-Ester"**, The Journal of Antibiotics, 27(3), 155-160, 1974.
- Loken, M.R., Sweet, R.G. and Haerzenberg, L.A. **"Cell Discrimination by Multiangle Light Scattering"**, The Journal of Histochemistry and Cytochemistry, 24(1), 284-291, 1976.
- Lopez, J. and Fortnagel, P. **"The Regulation of the Butanediol Cycle in *Bacillus Subtilis*"**, Biochimica et Biophysica Acta, 279, 554-560, 1972.
- Ludeking R. and Piret, E.L. **"A Kinetic Study of the Lactic Acid Fermentation. Batch Process at Controlled pH"**, Journal of Biochemical and Microbiological Technology and Engineering, 1(4), 393-412, 1959.
- Ludeking R. and Piret, E.L. **"Transient and Steady States in Continuous Fermentation. Theory and Experiment"**, Journal of Biochemical and Microbiological Technology and Engineering, 1(4), 431-459, 1959b.
- Luli, G., Jeannotte, A. and Hatch, R.T. **"Use of an Optical Density Probe for the Monitoring and Control of Fed-Batch Fermentations"**, Society of Industrial Microbiology Meeting, Seattle, August, 1989.

Lunde, G. "Activation Analysis of Trace Elements in Fishmeal", Journal of Sci. Fd. Agric., 19, 432-434, 1968.

Maaløe, O. "The International Reference Preparation for Opacity", Bulletin of the World Health Organization, 12, 769-775, 1955.

Mahmoud, S.A.Z., Taha, S.M. and Ali, S.A. "Production of 2,3-Butanediol by Local Bacterial Strains: III. Effect of Carbon and Nitrogen Components", Journal of Microbiology of the United Arab Republic, 6(1-2), 73-81, 1971.

Mahmoud, S.A.Z., Taha, S.M., El-Sadek, G.M. and Ali, S.A. "Effect of Some Carbon and Nitrogen Sources on the Production of 2,3-Butanediol", Rev. Microbiol. (S. Paulo), 6(4), 77-80, 1975.

Marin-Iniesta, F. "New Method for the Characterization of the Microbial Growth in Carrageenan Gel", Acta Biotechnol., 9(1), 55-62, 1989.

Marison, I.W. and von Stokar, U. "The Application of a Novel heat Flux Calorimeter for Studying Growth Of *Escherichia Coli* W in Aerobic Batch Culture", Biotechnology and Bioengineering, 28, 1780-1793, 1986.

Mas, C.de, Jansen, N.B. and Tsao, G.T. "Production of Optically Active 2,3-Butanediol by *Bacillus Polymyxa*", Biotechnology and Bioengineering, 31, 366-377, 1988.

May, B.K. and Elliott, W.H. "Characteristics of Extracellular Protease Formation by *Bacillus subtilis* and its Control by Amino Acids", Biochimica et Biophysica Acta, 157, 607-615, 1968.

Mayevsky, A. and Chance B. "Intracellular Oxidation-Reduction State Measured in situ by a Multichannel Fiber-Optic Surface Fluorimeter", Science, 217, 537-540, 1982.

McLaren, A.D. and Packer, L. "Some Aspects of Enzyme Reactions in Heterogeneous Systems", Advances in Enzymology and Related Areas of Molecular Biology, Volume 33, Edited by F.F. Nord, 245-308, Interscience Publishers, New York, 1970.

McMillan, J.D. "Mechanisms of Oxygen Transfer Enhancement During Submerged Cultivation in Perfluorochemical-in-Water Dispersions", PhD thesis, Massachusetts Institute of Technology, 1990.

Meltzer, Y.L. "Protease", Chapter 15, Encyclopedia of Enzyme Technology, Future Stochastic Dynamics, Inc., P.O. Box 5828, Station A, Flushing, New York 11358, 1973.

Meynell, G.G. and Meynell, E. "Theory and Practice in Experimental Bacteriology", Second Edition, University Press, Cambridge, 1970.

Miller, T.L. and Churchill, B.W. **"Substrates for Large-Scale Fermentations"**, Chapter 10 in **Manual of Industrial Microbiology and Biotechnology**, edited by A.L. Demain and N.A. Solomon, American Society for Microbiology, Washington DC, 1986.

Mink, R.W. and Dugan, P.R. **"Tentative Identification of Methanogenic Bacteria by Fluorescence Microscopy"**, *Applied and Environmental Microbiology*, 33(3), 713-717, 1977.

Moes, J., Griot, M., Keller, J., Heinzle, E., Dunn, I.J. and Bourne, J.R. **"A Microbial Culture With Oxygen Sensitive Product Distribution as a Potential Tool for Characterizing Bioreactor Oxygen Transport"**, 27, 482-489, 1985.

Monod, J. **"The Growth of Bacterial Cultures"**, *Annual Review of Microbiology*, Volume 3, 371-394, Edited by C.E. Clifton, S. Raffel, and H.A. Barker, Annual Reviews Inc., 1949

Moreira, A.R., Phillips, J.A., and Humphrey, A.E. **"Method for Determining the Concentration of Adsorbed Protein and Cell Biomass in Cellulose Fermentations"**, *Biotechnology and Bioengineering*, 20, 1501-1505, 1978.

Morris, v.J. and Jennings, B.R. **"Light Scatter by Bacteria: I Angular Dependence of the Scattered Light"**, *Proceedings of the Royal Society of London*, 338, 197-208, 1974.

Motai, H., Fukushima, Y., Fukase, T. and Itoh, H. **"Process for Producing Protease by Cultivating a Protease-Producing Mold in a Liquid Medium"**, US Patent, 4,879,235; 1989.

Murao, S. **"Production of a New Protease"**, US Patent, 3,622,458; 1971.

Neumark, R. and Citri, N. **"Repression of Protease Formation in *Bacillus Cereus*"**, *Biochimica Biophysica Acta*, 59, 749-751, 1962.

Nijenhuis, B.te **"Alkaline Protease Produced by a *Bacillus*"**, US patent 4,002,572; 1977.

Noe, A.F. and Beckhorn, E.J. **"Debridement Agent"**, US patent, 3,409,719; 1968.

Norris, J.R., Berkeley, R.C.W., Logan, N.A. and O'Donnell, A.G. **"The Genus *Bacillus* and *Sporolactobacillus*"**, Chapter 135, Volume 2, *The Prokaryotes, A Handbook on Habitats, Isolation and Identification of Bacteria*, Edited by M.P. Starr, H. Stolp, H.G. Truper, A. Balows, and H.G. Schlegel, Springer-Verlag, 1981.

O'Connor, G.M. **"Development of an Intelligent Fermentation Control System"**, PhD thesis, Massachusetts Institute of Technology, 1989.

Ogrydziak, D.M., Demain, A.L. and Tannenbaum, S.R. **"Regulation of Extracellular Protease Production in *Candida Lipolytica*"**, *Biochimica et Biophysica Acta*, 497, 525-538, 1977.

Old, R.W. and Primrose, S.B. **"Principles of Gene Manipulation: An Introduction to Genetic Engineering"**, Studies in Microbiology Volume 2, Third Edition, Blackwell Scientific Publications, Boston, 1985.

Omstead, D.R. Phillips, J.A. and Humphrey, A.E. **"Indirect Parameter Estimation"**, Chapter 4 in Computer Control of Fermentation Processes, edited by D.R. Omstead, CRC Press Inc., Boca Raton, Florida, 1990.

Onken, U., Buchholz, R. and Sittig, W. **"Measurement and Instrumentation"**, Chapter 29 in volume 2 of Biotechnology, edited by H.-J. Rehm and G. Reed, VCH, Weinheim, 1985.

Patterson-Curtis, S.I. and Johnson, E.A. **"Regulation of Neurotoxin and Protease Formation in *Clostridium botulinum* Okra B and Hall A by Arginine"**, Applied and Environmental Microbiology, 55(4), 1544-1548, 1989.

Pazlarova, J. and Tsaplina, I. **"Production of an Extracellular Proteinase and α -Amylase by *Bacillus subtilis* in Chemostat"**, Folia Microbiol., 33, 267-272, 1988.

Phillips, J.A. **"Novel Sensors for the Monitoring of Fermentation Processes"**, Chapter 2 in Computer Control of Fermentation Processes, edited by D.R. Omstead, CRC Press Inc., Boca Raton, Florida, 1990.

Pirt, S.J. **"Principles of Microbe and Cell Cultivation"**, Blackwell Scientific Publications, Boston, 1975.

Powell, E.O. **"Photometric Methods in Bacteriology"**, Journal of the Science of Food and Agriculture, 14, 1-7, 1963.

Powell, J.F. **"Factors Affecting the Germination of Thick Suspensions of *Bacillus subtilis* Spores in L-Alanine Solution"**, Journal of General Microbiology, 4(3), 330-338, 1950.

Powell, M. and Kennedy, M.J. **"The Utilization of Solid Substrate in Submerged Culture Fermentations"**, REU Program Report, Summer, 1988.

Priest, F.G. **"Extracellular Enzyme Synthesis in the Genus *Bacillus*"**, Bacteriological Reviews, 41(3), 711-753, 1977.

Rahaman, R. S., Chee, J. Y., Cabral, J. M. S. and Hatton, T. A. **"Recovery of an Extracellular Alkaline Protease From Whole Fermentation Broth Using Reversed Micelles"**, Biotechnology Progress, 4(4), 218-224, 1988.

Raimbault, M. and Alazard, D. **"Culture Method to Study Fungal Growth in Solid Fermentation"**, European Journal of Applied Microbiology and Biotechnology, 9, 199-209, 1980.

Ramirez , W.F. and Park, S. **"On-Line Measurement of Cell Density and Glucose Concentration in Bioreactors"**, Division of Biochemical Technology 117, American Chemical Society, 199th National Meeting, Boston, Massachusetts, April 22-27, 1990.

Ramsey, W.S., Nowlan, E.D., Simpson, L.B., Messing, R.A. and Takeguchi, M.M. **"Applications of Fluorophore-Containing Microbial Growth Media"**, Applied and Environmental Microbiology, 39(2), 372-375, 1980.

Rao, D.R. and Kamasastri, P.V. **"Quality of Fishmeal Processed from Sun-Dried Fish in a Commercial Fishmeal Dryer"**, Fishery Technology, 8(1), 51-54, 1971.

Ratledge, C. **"Fermentation Substrates"**, Chapter 3 in Volume 1 of Annual reports on Fermentation Process, edited by D. Perlman and G.T. Tsao, Academic Press, New York, 1977.

Reed, G. and Underkofler, L.A. **"Proteolytic Enzymes"** Chapter 7 and **"Production of Commercial Enzymes"**, Chapter 10, Enzymes and Food Processing, Academic Press, New York, 1966.

Reis, M.A.M., Lemos, P.C., Almeida, J.S. and Carrondo, M.J.T. **"Influence of Produced Acetic Acid on Growth of Sulfate Reducing Bacteria"**, Biotechnology Letters, 12(2), 145-148, 1990.

Sato, K., Nagatani, M., Nakamura, K.-I. and Sato, S. **"Growth Estimation of *Candida lipolytica* from Oxygen Uptake in a Solid State Culture with Forced Aeration"**, Journal of Fermentation Technology, 61(6), 623-629, 1983.

Schaeffer, P., Millet, J. and Aubert, J.P. **"Catabolic Repression of Bacterial Sporulation"**, Proceedings of the National Academy of Sciences, 54, 704-711, 1965.

Schaffeld, G., Bruzzone, P., Illanes, A., Curotto, M. and Aguirre, C. **"Enzymatic Treatment of Stickwater from Fishmeal Industry with Protease from *Cucurbita ficifolia*"**, Biotechnology Letters, 11(7), 521-522, 1989.

Schaffeld, G. and Illanes, A. **"Determination of Cell Mass in an Insoluble lignocellulosic Substrate in Submerged Fermentation"**, Biotechnology Letters, 4(10), 667-672, 1982.

Schuler, M.L. and Tsuchiya, H.M. **"Determination of Cell Numbers and Cell Size for *Thiobacillus ferrooxidans* by Electrical Resistance Method"**, Biotechnology and Bioengineering, 17, 621-624, 1975.

Scullard, G. and Meynell, E. **"Bacterial Mass Measured with the MCR Grey-Wedge Photometer"**, Journal of Pathology and Bacteriology, 91, 608-612, 1966.

Sen, D.P. and Keshava, N. **"Certain Observations on the Analysis of Fishmeal"**, Indian food Packer, 21-27, September-October, 1971.

Sen, D.P., Sripathy, N.V., Lahiry, N.L., Sreenivasan, A. and Subrahmanyam, V. **"Fish Hydrolysates: I. Rate of Hydrolysis of Fish Flesh with Papain"**, Food Technology, 138-141, May, 1962.

Shabtai, Y. and Wang, D.I.C. **"Production of Emulsan in a Fermentation Process Using Soybean Oil (SBO) in a Carbon-Nitrogen Coordinated Feed"**, Biotechnology and Bioengineering, 35, 753-765, 1990.

Smythe, C.V., Drake, B.B. and Neubeck, C.E. **"Production of Enzymes in Submerged Cultures of Bacteria"**, US patent, 2,530,210; 1950.

Sneath, P.H.A. **"Endospore-Forming Gram-Positive Rods and Cocci"**, Section 13, Bergey's Manual of Systematic Bacteriology, Volume 2, Edited by P.A.H. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt, Williams and Wilkins, Baltimore, 1986.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J. G. **"Bergey's Manual® of Systematic Bacteriology"**, Volume 2, p1130, Williams and Wilkins, Baltimore, 1986.

Solomon, B.O., Erickson, L.E. and Yang, S.S. **"Estimation of Biomass Concentration in the Presence of Solids for the Purpose of Parameter Estimation"**, Biotechnology and Bioengineering, 25, 2469-2477, 1983.

Sripathy, N.V., Sen, D.P., Lahiry, N.L., Sreenivasan, A. and Subrahmanyam, V. **"Fish Hydrolysates: II. Standardization of Digestion Conditions for Preparation of Hydrolysates Rich in Peptones and Proteoses"**, Food Technology, 141-142, May, 1962.

Starace, C.A. **"Detergent Enzymes: Developments During the Last Decade"**, JAOCS, 165a-170a, 1981.

Starace, C. and Barfoed, H. **"Enzyme Detergents"**, Volume 9, Kirk-Othmer Encyclopedia of Chemical Technology, Third Edition, John Wiley and Sons, New York, 1980.

Steffan, R.J., Breen, A., Atlas, R.M. and Sayler, G.S. **"Application of Gene Probe Methods for Monitoring Specific Microbial Populations in Freshwater Ecosystems"**, Canadian Journal of Microbiology, 35, 681-685, 1989.

Stull, V.R. **"Size Distribution of Bacterial Cells"**, Journal of Bacteriology, 109(3), 1301-1303, 1972.

Suzuki, T., Government Industrial Research Institute, Nagoya Agency of Industrial Science and Technology, Ministry of International trade and Industry, Hirate-cho, Kita-ku, Nagoya,

Japan, private communications, 1988.

Suzuki, T., Yamane, T. and Shimizu, S. **"Control of Carbon Source Supply and Dissolved Oxygen by Use of Carbon Dioxide Concentration of Exhaust Gas in Fed-Batch Culture"**, Journal of Fermentation Technology, 64(4), 317-326, 1986.

Takamine, J. **"Preparing and Making Taka-Koji"**, US Patent, 525,820; 1894.

Tang, I.-C., Okos, M.R. and Yang, S.-T. **"Effects of pH and Acetic Acid on Homoacetic Fermentation of Lactate by *Clostridium formicoaceticum*"**, Biotechnology and Bioengineering, 34, 1063-1074, 1989.

Tateishi, T., Horikoshi, T., Tsubota, H. and Takahashi, F. **"Application of the Chloroform Fumigation-Incubation Method to the Estimation of Soil Microbial Biomass in Burned and Unburned Japanese Red Pine Forests"**, FEMS Microbiology Ecology, 62, 163-172, 1989.

Tengerdy, R.P. **"Solid Substrate Fermentation"**, Trends in Biotechnology, 3(4), 96-99, 1985.

Thierry, A. and Chicheportiche, R. **"Use of ATP Bioluminescence Measurements for the Estimation of Biomass during Biological Humidification"**, Applied Microbiology and Biotechnology, 28, 199-202, 1988.

Tsen, H.-Y., Chen, M.-H., Shieh, J.-S., Wang, S.-J. and Hu, N.-T. **"Possible Use of a 1.8 kb DNA Fragment for the Specific Detection of *Salmonella* in Foods"**, Journal of Fermentation and Bioengineering, 68(1), 1-6, 1989.

Venugopal, V., Alur, M.D. and Nerkar, D.P. **"Solubilization of Fish Proteins Using Immobilized Microbial Cells"**, Biotechnology and Bioengineering, 33, 1098-1103, 1989.

Viccaro, J.P. **"Alkaline Protease, Method for its Production, and Detergent Composition"**, US patent, 3,748,233; 1973.

Wang, D.I.C. and Cooney, C.L., Biochemical Engineering Course, 10.59, Massachusetts Institute of Technology, 1986.

Wang, D.I.C., Cooney, C.L., Demain, A.L., Dunnill, P., Humphrey, A.E., and Lilly, M. D. **"Fermentation and Enzyme Technology"**, John Wiley and Sons, New York, 1979.

Wang, G. and Wang, D.I.C. **"Elucidation of Growth Inhibition and Acetic Acid Production by *Clostridium thermoaceticum*"**, Applied and Environmental Microbiology, 47(2), 294-298, 1984.

Wang, L.-F., Hum, W.T., Kalyan, N.K., Lee, S.G., Hung, P.P. and Doi, R.H. **"Synthesis and Refolding of Human Tissue-Type Plasminogen Activator in *Bacillus subtilis*"**, Gene, 84, 127-

133, 1989.

Ward, O.P. "**Proteinases**", Chapter 6, Microbial Enzymes and Biotechnology, Edited by W.M. Fogarty, Applied Sciences Publishers, New York, 1983.

Ward, O.P. "**Proteolytic Enzymes**", Comprehensive Biotechnology, Volume 3, The Practice of Biotechnology: Current Commodity Products, Edited by H.W. Blanch, S. Drew and D.I.C. Wang, Chapter 40, 789-818, Pergamon Press, New York, 1985.

Wei, C.J., Tanner, R.D., Malaney, G.W. and Charles, M. "**An On-Line Indirect Measurement Technique for Monitoring Yeast Cell Biomass in Semi-Solid Gels**", Process Biochemistry, March/April, 1983.

Welker, N.E. and Campbell, L.L. "**Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis***", Journal of Bacteriology, 94(4), 1124-1130, 1967.

West, W. "**Absorption of Electromagnetic Radiation**", Volume 1, McGraw-Hill Encyclopedia of Science and Technology, 5th edition, 15-21, McGraw-Hill Book Company, New York, 1982.

Williamson, D. H. and Fennell, D. J. "**The Use of Fluorescent DNA-Binding Agent for Detecting and Separating Yeast Mitochondrial DNA.**", Methods in Cell Biology, 12, 335-351, 1975.

Wittrup, K.D., Mann, M.B., fenton, D.M., Tsai, L.B. and Bailey, J.E. "**Single-Cell Light Scatter as a Probe of Refractile Body Formation in *Escherichia Coli***", Biotechnology, 6, 423-426, 1988.

Wyatt, P.J. "**Scattering of Electromagnetic Plane Waves from Inhomogeneous Spherically Symmetric Objects**", Physical Review, 127(5), 1837-1843, 1962.

Wyatt, P.J. "**Identification of Bacteria by Differential Light Scatter**", Nature, 221, 1257-1258, 1969.

Wyatt, P.J. "**Cell Wall Thickness, Size distribution, Refractive Index Ratio and Dry Weight Content of Living Bacteria (*Staphylococcus aureus*)**", Nature, 226, 277-279, 1970.

Wyatt, P.J. "**Light Scattering in the Microbial World**", Journal of Colloid and Interface Science, 39(3), 479-491, 1972.

Wyatt, P.J. and Phillips, D.T. "**Structure of Single Bacteria from Light Scattering**", Journal Theor. Biol., 37, 493-501, 1972.

Yamada, K. and Yogo, M. "**Studies on the Utilization of Hydrocarbons by Microorganisms:**

Part 15: Yeast and Bacterial Cell Production from Paraffin Wax", Agricultural and Biological Chemistry, 34(2), 296-301, 1970.

Yeh, T.Y., Godshalk, J.R., Olson, G.J. and Kelly, R.M. "Use of Epifluorescence Microscopy for Characterizing the Activity of *Thiobacillus ferrooxidans* on Iron Pyrite", Biotechnology and Bioengineering, 30, 138-146, 1987.

Zabriskie, D.W., Armiger, W.B., Phillips, D.H. and Albano, P.A. "Traders' Guide to Fermentation Media Formulation", Traders Protein, Memphis, Second Edition, 1982.

APPENDIX A

FORTRAN Data Acquisition and Control Programs.

The fortran programs to collect data and control the fermenter were comprised of the following programs and subprograms:

- 1) MKMAIN: This is the main program which contains the iterative loop for each time step.
- 2) MKDATA: This subprogram is the pièce de résistance and contains all the control strategies, file handling, data storage and data display.
- 3) MKTIM: This subprogram calculates the elapsed time.
- 4) MKINIT: This program is run before the fermentation to initialize the system.
- 5) MKCHNG: This program is used to change parameters during the fermentation.
- 6) MKPRNT: This program prints a hardcopy of the data after or during the fermentation.
- 7) MKPLOT: This program plots the data to the screen during or after the fermentation.
- 8) CHG080: MKPRNT subprogram which changes the output of the printer to 80 characters per line.
- 9) CHG132: MKPRNT subprogram which changes the output of the printer to 132 characters per line.
- 10) VOLOUT: Subprogram which addresses the TCS signal conditioning unit.
- 11) FPLT3: MKPLOT subprogram which plots the data.

The author gratefully acknowledges a number of previous students programming efforts upon which this program was built.

```

PROGRAM MKMAIN
C
C PROGRAM TO MONITOR COMP. OF EXIT GAS FROM FERMENTOR.
C TCS DATA
C
C MODIFIED TO USE TCS TO MEASURE D.O., PH, AND GLUCOSE LOAD CELL C MARCH, 1986.....ALSO
OPTION TO MEASURE GAS FLOW RATE VIA TCS...
C
C*****
C Modified June, 1985, by Jim McMillan from Dave Robinson's DRMAIN
C program
C*****
C Modified August, 1986, by Max Kennedy from Jim McMillan's JDMAIN
C*****
C Modified January, 1988, by Max Kennedy
C Purpose: The new protease programme
C*****
C
C SEE SUBROUTINE MKDATA FOR AN EXPLANATION OF VARIABLES
C
C*****
C BYTE ADATE(9),ATIME(8)
C BYTE BATCHN(6),DATFIL(24),PRMFIL(24),CLCFIL(24),TCSFIL(24)
C BYTE GLUFIL(24)
C INTEGER AIRFLG
C REAL NH3INL,NH3EXT,KLA
C
C COMMON/FILNAM/ DATFIL,PRMFIL,CLCFIL,TCSFIL,GLUFIL
C COMMON/TIMES/ ETIME
C COMMON/INCTIM/ IYEAR,IMON,IDAY,IHR,IMIN,ISEC
C
C COMMON/TCS101/ BATCHN,SCNINT,ISCAN,MSEXCN,MSINCN,AQVOL,
& AIRF,AIRFLG,IREC,TLAST,PFERM,
& DO2CON,AINOC,AGLUC,SLOPE,AINTER,ACELL,APUMP
C
C COMMON/TCS103/ VOLCO2,TOTCO2,TOTO2,SGLU,FGLU
C
C PREVENT OUTPUT OR CONVERSION ERRORS FROM STOPPING THE PROGRAM
CALL ERRSET (63,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (70,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (71,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (72,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (73,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (74,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (75,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (83,.TRUE.,.FALSE.,,.FALSE.,500)
C
C DATA PRMFIL /'D','L','1',:,'[','3','1','4',:,'0','4','5',:],
& 'X','X','X','X','X','X',:,'P','R','M',0/
C DATA DATFIL /'D','L','1',:,'[','3','1','4',:,'0','4','5',:],
& 'X','X','X','X','X','X',:,'D','T','M',0/

```

```

DATA CLCFIL /'D','L','1',:,:['3','1','4',:,:0','4','5'],
&      'X','X','X','X','X','X',:,'D','T','C',0/
DATA TCSFIL /'D','L','1',:,:['3','1','4',:,:0','4','5'],
&      'X','X','X','X','X','X',:,'T','C','S',0/
DATA GLUFIL /'D','L','1',:,:['3','1','4',:,:0','4','5'],
&      'X','X','X','X','X','X',:,'G','L','U',0/
C

DO 57 J=14,19
DATFIL(J)=BATCHN(J-13)
CLCFIL(J)=BATCHN(J-13)
PRMFIL(J)=BATCHN(J-13)
GLUFIL(J)=BATCHN(J-13)
57  TCSFIL(J)=BATCHN(J-13)
OPEN(UNIT=1,TYPE='OLD',ACCESS='DIRECT',
&      NAME=PRMFIL,RECL=16)
READ(1'1) ILAST,ETIME
READ(1'2) IYEAR,IMON,IDAY,IHR,IMIN,ISEC
CALL DATE (ADATE)
CALL TIME (ATIME)
WRITE(6,58) ADATE,ATIME
58  FORMAT(/' ',9A1,2X,8A1)
WRITE(6,60) BATCHN,SCNINT,ISCAN,MSEXCN,MSINCN,
&      AQVOL,FCVOL,AIRF
60  FORMAT (5X,6A1,F5.1,3I5,3F8.2)

CLOSE(UNIT=1)
IREC=ILAST+1
C
90  CONTINUE
C
C THE FOLLOWING STATEMENT ALLOWS MKMAIN TO BE AUTOMATICALLY RESTARTED--
C WITHOUT RUNNING MKINIT OR MKCHNG--IN THE EVENT OF A SYSTEM CRASH OR
C IF THE NUMBER OF SCANS EXCEEDS THE MAXIMUM NUMBER OF SCANS, ISCAN.
C
ISCAN=ISCAN+ILAST
C
C***** BEGIN DATA SCAN LOOP. ONE SCAN PER SCAN INTERVAL, SCNINT.*****
C
100 CONTINUE
C
ISCNSC=SCNINT*60.
CALL MARK(1,ISCNSC,2,IDS)
IF(IDS.EQ.1) GO TO 110
CALL DATE (ADATE)
CALL TIME (ATIME)
WRITE(5,108) ADATE,ATIME,IDS
108 FORMAT(/' ',9A1,2X,8A1,' MKMAIN: MARK TIME FAILURE. IDS=',I2)
110 CONTINUE
C
C

```

```
      CALL MKTIM
C
      CALL MKDATA
C
      CALL STOPFR(1,IDS)
C
      IREC=IREC+1
      IF(IREC.GE.ISCAN) GO TO 2000
      GO TO 100
C
C ***** CONCLUDE SCAN LOOP. ONE INTERVAL ELAPSED*****
C
2000  CONTINUE
      STOP
      END
```

SUBROUTINE MKDATA

```

C
C PRINT LINE OF DATA FOR EVERY SCAN INTERVAL
C
C Modified to include TCS data March, 1986
C
C*****
C Modified June, 1985, by Jim McMillan from Dave Robinson's DRDATA
C program
C*****
C Modified August, 1986, by Max Kenndy from Jim McMillan's JDDATA
C Changes:
C   1, Deletion of Jim's glucose control strategy
C   2, Inclusion of control strategy for solid oxygen storage
C      materials project. ppO2 in the gas phase above the fermenter
C      equals the ppO2 in the liquid phase. Option included to
C      either have no control, set the gas flowrates or controlled
C      gas flowrates
C   3, Modification of printout of the data both to the screen and
C      files. Variables associated with 2 included and DO reading
C      and scan rate sent to the screen.
C   4, TCS common changed from TCS105 to Firoz's old common TCS101
C
C Variables added:
C   ICOUN : control strategy flag
C   ASET  : set value of air flow (lpm)
C   ANSET : set value of nitrogen flow (lpm)
C   ACON  : conversion factor air flow/volts
C   ANCON : conversion factor nitrogen flow/volts
C   AIRFLO: return signal from air valve (volts)
C   ANFLO : return signal from nitrogen valve (volts)
C   AAIR  : airflo value converted to lpm
C   ANITRO: anflo value converted to lpm
C   THEOPC: theoretical percent O2 in mixed streams
C   VASET : aset value converted to volts
C   VANSET: anset value converted to volts
C   VOLCO2: total co2 produced per liter (time weighted)
C   ADDCEL: total grown cell mass
C   SLOPE : slope of the total co2 to cell mass curve
C   AINTER: intercept of the total co2 to cell mass curve
C   AINOC  : cell mass conc of the inoculated vessel
C   CELL  : cell mass conc in the vessel
C   ACELL : cell mass produced per glucose utilized (g/g)
C   AUSED : total glucose used (g/l)
C   AON   : total minutes the glucose feed pump should be on
C   AGLUC : initial glucose concentration
C   APUMP : total minutes the glucose feed pump has been on
C   ZGLU  : glucose concentration
C   CELLST: cell concentration at the last time interval
C   ARATE : specific growth rate (1/h)
C   ARUN  : time left for glucose exhaustion if no more

```



```

C          glucose is fed
C      DIFF : difference between pump should have been on
C          and pump actually on
C      SGLU : glucose set point (g/l)
C      FGLU : glucose concentration of feed stream (g/l)
C      ZFACT : glucose feed stream addition rate usually 6.72
C              for 5 l working volume
C              (g glucose/l fermenter working vol-h)
C
C Variables deleted:
C      TOTCO2
C      GLULDO
C      GLUCON
C      AIRCON
C
C Added Subroutines
C      VOLOUT: from Greg O'Connor, converts volts to
C              hexadecimal ascii output and puts the values
C              into any specified TCS location.
C      CONVDH: used within VOLOUT and is stored in
C              DL0:[5,2]CONVDH don't forget to include
C              this within your TKB file
C
C*****
C
C Calibration data: Valves calibrated 22 Aug 1986
C
C      Air Valve: FLOW=3.814*SIGNAL+1.4
C                  SIGNAL=0.262*FLOW-0.367
C
C      Nitrogen Valve: FLOW=3.98*SIGNAL+1.0
C                      SIGNAL=0.251*FLOW-0.251
C
C      Where: FLOW = flowrate (l/min)
C              SIGNAL= signal (volts)
C
C*****
C
C EXPLANATION OF VARIABLES:
C
C PARAMETERS THAT ARE CALCULATED:
C
C      OUR   = Oxygen (O2) uptake rate          (mMol/L-hr)
C      CO2ER = Carbon dioxide (CO2) evolution rate (mMol/L-hr)
C      KLA   = Overall volumetric mass transfer
C              coefficient for oxygen transfer
C              based on partial pressure driving
C              force (actually KLA/H, where H is
C              Henry's Law Constant)          (mMol/L-hr-atm)
C
C      Note: These rates are determined on a per unit volume

```

C aqueous phase basis. Calculations assume constant
 C temperature (25 degrees Celcius). This assumption
 C underlies the 24.54 constant used in the calcuations.
 C
 C RSPQUO = Respiration coefficient = CO2ER/OUR (dimensionless)
 C TOTO2 = Total O2 consumed (mMol)
 C TOTCO2 = Total CO2 evolved (mMol)
 C PLIQUO2 = Liquid phase partial pressure of O2 (atm)
 C PGASO2 = Gas phase partial pressure of O2 (atm)
 C GLUFED = Total glucose fed (grams)
 C VOLPCT = Volume percent of dispersed phase (dimensionless)
 C
 C PARAMETERS THAT ARE INPUT MANUALLY:
 C
 C AQVOL = Aqueous (continuous phase) volume (L)
 C FCVOL = Immiscible (dispersed phase) volume (L)
 C AIRF = Air flow rate into vessel (L/min)
 C AIRFLG = Integer specifying how air flow is
 C to be measured. If AIRFLG = 0
 C then airflow is determined by the
 C TCS unit. Otherwise it is input
 C manually. Note AIRFLG IS AN INTEGER
 C
 C Note: This air flow rate should be corrected to 1 atm
 C absolute
 C
 C PFERM = Vessel operating pressure (psia)
 C ISCAN = Maximum number of scans desired (integer)
 C SCNINT = Scan interval (min)
 C
 C CONVERSION FACTORS USED TO CONVERT TCS SIGNALS
 C
 C GLUCON = conversion factor for glucose load
 C cell (grams glucose per volt output) (grams/V)
 C DO2CON = conversion factor for D.O. output
 C (atm O2 per volt output) (atm/V)
 C
 C PARAMETERS INVOLVING THE MASS SPECTROMETER INPUTS:
 C
 C Note: Gas compositions are given in percent form
 C
 C 'EXT' = Exit gas suffix
 C 'INL' = Inlet gas suffix
 C
 C Note: The prefix 'A' is used at times to make the
 C otherwise self-descriptive gas variable names
 C 6 characters in length
 C
 C *****
 C
 C Modified January, 1988, by Max Kennedy.

```

C For work on the protease project.
C Major change was the deletion of the gas flow controller sections
C and the display of more of the mass spec data
C
C*****
      BYTE DATFIL(24),PRMFIL(24),CLCFIL(24),TCSFIL(24),BATCHN(6)
      BYTE GLUFIL(24)
      BYTE CTIME(8),CDATE(9)
      INTEGER CYEAR,CMON,CDAY,CHR,CMIN,CSEC,AIRFLG
      REAL NH3INL,NH3EXT,KLA
      COMMON/FILNAM/ DATFIL,PRMFIL,CLCFIL,TCSFIL,GLUFIL
      COMMON/TIMES/ ETIME
      COMMON/DATTIM/ CDATE,CTIME
      COMMON/CTIM/ CYEAR,CMON,CDAY,CHR,CMIN,CSEC

      COMMON/TCS101/ BATCHN,SCNINT,ISCAN,MSEXCN,MSINCN,AQVOL,
&          AIRF,AIRFLG,IREC,TLAST,PFERM,
&          DO2CON,AINOC,AGLUC,SLOPE,AINTER,ACELL,APUMP
C
C      COMMON/TCS103/ VOLCO2,TOTCO2,TOTO2,SGLU,FGLU
C
C      INCLUDE 'DL0:[5,5]MS0.COM'
C
C      INCLUDE 'DL0:[5,5]MS2.COM'
C
C      INCLUDE 'DL0:[5,5]TCS000.COM'
C
C      INCLUDE 'DL0:[5,5]TCS002.COM'
C
C      INCLUDE 'DL0:[5,5]TCSA14.COM'
C
C      INCLUDE 'DL0:[5,5]TCSDO4.COM'
C
C
C EQUIVALENCE STATEMENTS FOR 2 LITER SETRIC FERMENTER
C
C      EQUIVALENCE(DISSO2,DPVAI(1,3)),(PH,DPVAI(2,3))
      EQUIVALENCE(RLOAD1,DPVAI(8,3)),(RFLOW1,DPVAI(4,3))
      EQUIVALENCE(AIRFLO,DPVAI(5,3))
      EQUIVALENCE(ANFLO,DPVAI(6,3))
C
C      PREVENT OUTPUT OR CONVERSION ERRORS FROM STOPPING THE PROGRAM
      CALL ERRSET (63,.TRUE.,.FALSE.,,.FALSE.,500)
      CALL ERRSET (70,.TRUE.,.FALSE.,,.FALSE.,500)
      CALL ERRSET (71,.TRUE.,.FALSE.,,.FALSE.,500)
      CALL ERRSET (72,.TRUE.,.FALSE.,,.FALSE.,500)
      CALL ERRSET (73,.TRUE.,.FALSE.,,.FALSE.,500)
      CALL ERRSET (74,.TRUE.,.FALSE.,,.FALSE.,500)
      CALL ERRSET (75,.TRUE.,.FALSE.,,.FALSE.,500)
      CALL ERRSET (83,.TRUE.,.FALSE.,,.FALSE.,500)

```

```

C
C
C EXIT GAS COMPOSITION DETERMINED VIA MSSCAN PROGRAM
C
C
      AN2EXT=GAS(1,MSEXCN)
      AO2EXT=GAS(2,MSEXCN)
      CO2EXT=GAS(3,MSEXCN)
      H2OEXT=GAS(4,MSEXCN)
      ETHEXT=GAS(5,MSEXCN)
      AH2EXT=GAS(6,MSEXCN)
      NH3EXT=GAS(7,MSEXCN)

C INLET GAS COMPOSITION DETERMINED VIA MSSCAN PROGRAM

      AN2INL=GAS(1,MSINCN)
      AO2INL=GAS(2,MSINCN)
      CO2INL=GAS(3,MSINCN)
      H2OINL=GAS(4,MSINCN)
      ETHINL=GAS(5,MSINCN)
      AH2INL=GAS(6,MSINCN)
      NH3INL=GAS(7,MSINCN)

C
C CHECK IF MSSCAN PROPERLY OPERATING
C
      IF(AN2INL.LT.-10.0) GOTO 10
      IF(AN2INL.GT.110.0) GOTO 10
      IF(AO2INL.GT.110.0) GOTO 10

C
      IF(AN2EXT.LT.-10.0) GOTO 10
      IF(AN2EXT.GT.110.0) GOTO 10
      IF(AO2EXT.GT.110.0) GOTO 10

C
      GOTO 20

C
10  WRITE(5,15)ETIME,AN2INL,AO2INL,AN2EXT,AO2EXT
15  FORMAT(/,,' MS ERROR IN MKDATA AT ',F8.2,/,
&    '    N2 INLET = ',F8.2,5X,'O2 INLET = ',F8.2,/,
&    '    N2 EXIT  = ',F8.2,6X,'O2 EXIT = ',F8.2)

C
C DECREMENT IREC AND RETURN TO MKMAIN IF MSSCAN FUNCTIONING IMPROPERLY
C
      IREC=IREC-1
      GOTO 200

C
20  CONTINUE

C
C CHECK WHICH VALUE FOR AIRFLOW IS TO BE USED IN THE CALCULATIONS
C RFLOW1 IS A DUMMY VARIABLE FOR AIRFLOW AS MEASURED BY THE TCS UNIT
C
      IF(AIRFLG.EQ.0)AIRF=RFLOW1

```

```

C
C DETERMINE OXYGEN UPTAKE AND CO2 EVOLUTION RATES
C NOTE: RATES ARE BASED ON UPTAKE PER UNIT VOLUME
C AQUEOUS PHASE USING THE INLET GAS FLOW RATE!!!!
C
  RATN2=AN2INL/AN2EXT
  OUR=24.54*(PFERM/14.7)*AIRF*(AO2INL-RATN2*AO2EXT)/AQVOL
  CO2ER=24.54*(PFERM/14.7)*AIRF*(RATN2*CO2EXT-CO2INL)/AQVOL
C
C DETERMINE RESPIRATION COEFFICIENT
C
  IF (OUR.LE.0.0) THEN
    OUR=0.0
    RSPQUO=9999.9
  ELSE
    RSPQUO=CO2ER/OUR
  END IF
C
C CALCULATE PARTIAL PRESSURE OF O2 IN THE LIQUID PHASE FROM TCS SIGNAL
C FOR DISSO2 (WHICH WILL BE A NUMBER BETWEEN 0 AND 10.00)
C
  PLIQO2=DO2CON*DISSO2
C
C CALCULATE GAS PHASE PARTIAL PRESSURE OF O2
C
  PGASO2=PFERM*(AO2EXT/100.)
C
C DETERMINE KLA (ACTUALLY KLA/H, WHERE H IS HENRY'S LAW CONSTANT)
C
  IF (DISSO2.GE.10.00) THEN
    KLA=0.0
  ELSE
    KLA=OUR/(PGASO2-PLIQO2)
  END IF
C
C CALCULATE GAS TOTALS
C
C totco2 taken out, leftover from Jim's glucose control programme
  TOTCO2=TOTCO2+CO2ER*(ETIME-TLAST)*AQVOL
  TOTO2=TOTO2+OUR*(ETIME-TLAST)*AQVOL
C
C glucose control strategy calculations
C
  VOLCO2=VOLCO2+CO2ER*(ETIME-TLAST)
  ZFACT=FGLU*1.4*60/1000/AQVOL
  ADDCEL=(VOLCO2-AINTER)/SLOPE
  CELL=AINOC+ADDCEL
  AUSED=ADDCEL/ACELL
  AON=(AUSED-(AGLUC-SGLU))/ZFACT*60
  ZGLU=AGLUC+APUMP/60*ZFACT-AUSED
  IF(TLAST.EQ.0) THEN

```

```

CELLST=AINOC
END IF
ARATE=(CELL-CELLST)/(ETIME-TLAST)/CELL
ARUN=ZGLU/(ARATE*CELL/ACELL)*60
IF(ARUN.GT.999999) THEN
ARUN=999999.0
END IF
IF(ARUN.LT.-999999) THEN
ARUN=-999999.0
END IF
DIFF=APUMP-AON
CELLST=CELL
TLAST=ETIME
C*****
C THE FOLLOWING SECTIONS HAVE BEEN COMMENTED OUT
C
C CALCULATE VOLUME PERCENT, VOLPCT, OF THE DISPERSED (OIL) PHASE
C
C   VOLPCT=100*FCVOL/(FCVOL+AQVOL)
C
C
C MAX'S NEW CONTROL PACKAGE
C
C
C CONVERTING RETURN SIGNAL FROM THE AIR AND NITROGEN FLOW CONTROLLER
C INTO lpm SIGNAL
C   AAIR=AIRFLO*ACON+1.4
C   ANITRO=ANFLO*ANCON+1.0
C
C
C CALCULATING THE THEORETICAL OXYGEN CONTENT OF THE MIXED GASES
C   THEOPC=AAIR*0.21/(AAIR+ANITRO)
C
C
C WARNING ERROR MESSAGE IF THE TCS UNIT CHANNEL 6, THE
C NITROGEN RETURN SIGNAL, DROPS TO ZERO WHEN THE SET
C VALUE IS NON-ZERO. I HAVE BEEN HAVING PROBLEMS WITH
C THIS CHANNEL FOR SOME TIME
C   DEL=0.001
C   IF(ANFLO.LT.DEL.AND.VANSET.GT.DEL) THEN
C     TYPE 401
C401   FORMAT(' WARNING! ERROR IN TCS UNIT')
C     TYPE 402
C402   FORMAT(' NITROGEN VALVE RETURN SIGNAL GONE TO ZERO')
C     TYPE 403
C403   FORMAT(' BUT THE NITROGEN VALVE SET POINT IS NON-ZERO')
C   END IF
C
C
C IF NO CONTROL THEN SET FLOWRATES TO ZERO
C   IF (ICOUN.EQ.1) THEN

```

```

C    VASET=0.262*ASET-0.367
C    VANSET=0.251*ANSET-0.251
C    CALL VOLOUT(3,2,VASET)
C    CALL VOLOUT(2,2,VANSET)
C    END IF
C
C
C
C SETTING OF THE AIR FLOWRATES
C    IF(ICOUN.EQ.2) THEN
C        GOTO 100
C    ELSE
C        GOTO 300
C    END IF
C
C
C
C CONVERTING LPM TO VOLTS FOR AIR AND NITROGEN SET POINTS
C100    VASET=0.262*ASET-0.367
C        VANSET=0.251*ANSET-0.251
C
C
C CHECKING VOLTAGE HIGH AND LOW RANGE SHOULD BE 0-5 VOLT SIGNAL
C    IF(VASET.GT.5) THEN
C        WRITE(5,101)ASET
C101    FORMAT(/,' ERROR - AIR FLOWRATE SET TOO HIGH',/,
C &        ' AIR FLOWRATE SET AT ',F6.2,' lpm',/)
C    END IF
C    IF(VASET.LT.0) THEN
C        WRITE(5,102)ASET
C102    FORMAT(/,' ERROR - AIR FLOWRATE SET TOO LOW',/,
C &        ' AIR FLOWRATE SET AT ',F6.2,' lpm',/)
C    END IF
C    IF(VANSET.GT.5) THEN
C        WRITE(5,103)ANSET
C103    ORMAT(/,' ERROR - NITROGEN FLOWRATE SET TOO HIGH',/,
C &        ' NITROGEN FLOWRATE SET AT ',F6.2,' lpm',/)
C    END IF
C    IF(VANSET.LT.0) THEN
C        WRITE(5,104)ANSET
C104    FORMAT(/,' ERROR - NITROGEN FLOWRATE SET TOO LOW',/,
C &        ' NITROGEN FLOWRATE SET AT ',F6.2,' lpm',/)
C    END IF
C
C
C
C CONVERTING VOLTAGE SIGNALS TO HEXIDECIMAL ASCII AND SENDING TO
C THE CORRECT CHANNEL OF THE TCS
C    CALL VOLOUT(3,2,VASET)
C    CALL VOLOUT(2,2,VANSET)
C300    CONTINUE
C
C
C CONTROL ALGORITHM

```

```

C    IF(ICOUN.EQ.3) THEN
C    GOTO 400
C    ELSE
C    GOTO 500
C    END IF
C400  IF(PLIQO2.LT.0.1) THEN
C    ANSET=10.
C    ASET=PLIQO2*ANSET/(0.21-PLIQO2)
C    ELSE
C    ASET=10.
C    ANSET=(0.21-PLIQO2)*ASET/PLIQO2
C    END IF
C
C
C TRANSFERRING SET VALUES TO THE CONTROLLER
C    VASET=0.262*ASET-0.367
C    VANSET=0.251*ANSET-0.251
C    CALL VOLOUT(3,2,VASET)
C    CALL VOLOUT(2,2,VANSET)
C
C
C END OF CONTROL SEGMENT
500  CONTINUE
C
C STORE DATA IN RECORD FILES FOR SUBSEQUENT ANALYSIS
C
C    OPEN(UNIT=1,TYPE='OLD',ACCESS='DIRECT',
C    &      NAME=PRMFIL,RECORDSIZE=16)
C
C    IF(IREC.NE.1) GO TO 50
C    OPEN(UNIT=2,TYPE='UNKNOWN',ACCESS='DIRECT',
C    &      NAME=DATFIL,RECORDSIZE=16)
C    OPEN(UNIT=3,TYPE='UNKNOWN',ACCESS='DIRECT',
C    &      NAME=CLCFIL,RECORDSIZE=16)
C    OPEN(UNIT=4,TYPE='UNKNOWN',ACCESS='DIRECT',
C    &      NAME=TCSFIL,RECORDSIZE=16)
C    OPEN(UNIT=7,TYPE='UNKNOWN',ACCESS='DIRECT',
C    &      NAME=GLUFIL,RECORDSIZE=16)
C
C    GO TO 60
C
C
50  OPEN(UNIT=2,TYPE='OLD',ACCESS='DIRECT',
C    &      NAME=DATFIL,RECORDSIZE=16)
C    OPEN(UNIT=3,TYPE='OLD',ACCESS='DIRECT',
C    &      NAME=CLCFIL,RECORDSIZE=16)
C    OPEN(UNIT=4,TYPE='OLD',ACCESS='DIRECT',
C    &      NAME=TCSFIL,RECORDSIZE=16)
C    OPEN(UNIT=7,TYPE='OLD',ACCESS='DIRECT',
C    &      NAME=GLUFIL,RECORDSIZE=16)
C
C
60  WRITE(1'1) IREC,ETIME

```



```

C
C
  WRITE(2'IREC) IREC,ETIME,AN2EXT,AO2EXT,CO2EXT,H2OEXT,
&      ETEXT,AH2EXT,NH3EXT,AN2INL,AO2INL,CO2INL,H2OINL,
&      ETHINL,AH2INL,NH3INL
C
  WRITE(3'IREC) IREC,CYEAR,CMON,CDAY,CHR,CMIN,CSEC,
&      ETIME,AQVOL,AIRF,OUR,CO2ER,TOTO2,TOTCO2,
&      RSPQUO
C
  WRITE(4'IREC) IREC,ETIME,PFERM,DO2CON,PLIQO2,PGASO2,KLA,
&      RFLOW1,CELL,AON,ZGLU,ARATE,ARUN,AUSED,
&      SCNINT,AIRFLG
C
  WRITE(7'IREC) AINOC,ACELL,SLOPE,AINTER,AGLUC,APUMP,DIFF,
&      SGLU,VOLCO2,FGLU
C
  CLOSE(UNIT=1)
  CLOSE(UNIT=2)
  CLOSE(UNIT=3)
  CLOSE(UNIT=4)
  CLOSE(UNIT=7)
C
C
C THE FOLLOWING IS THE PRINTOUT TO THE SCREEN
C
C FIRST COMES THE TIME DATA AND THE MEASURED DATA
C EXCEPT THE MASS SPEC DATA
C
  TYPE 300
300  FORMAT(/' TIME DATA, MEASURED DATA AND INITIATED VALUES')
  TYPE 85
85   FORMAT(/' REC# TIME',T12,' ELAP  SCAN  DO  AIR',
& ' AQUEOUS PUMP')
C
  TYPE 86
86   FORMAT(' ',T12,' TIME INT  LIQ  FLO',
& ' VOL  ACTUAL')
C
  TYPE 90, IREC,(CTIME(K),K=1,5),ETIME,SCNINT,PLIQO2,AIRF,
&      AQVOL,APUMP
C
90   FORMAT(' ',I4,1X,5A1,T12,F7.2,1X,F6.2,F6.2,1X,F6.2,
&      2X,F6.2,2X,F6.1,/)
C
C NEXT COMES THE MASS SPEC DATA
C
  TYPE 301
301  FORMAT ( ' MASS SPEC DATA',/)
  TYPE 302
302  FORMAT ( ' OXY    NITRO    CO2    H2O    CELL    GLUC    GLUC

```

```

& GLUC')
TYPE 303
303  FORMAT ( ' IN      IN      IN      IN      CONC  CONC  USED
& SET PT')
TYPE 304, AO2INL,AN2INL,CO2INL,H2OINL,CELL,ZGLU,AUSED,SGLU
304  FORMAT ( 4(F6.2,2X),3(F6.1,2X),F6.1,/)
TYPE 305
305  FORMAT ( ' OXY    NITRO    CO2    H2O    PUMP  GROWTH  RUN ')
TYPE 306
306  FORMAT ( ' OUT    OUT    OUT    OUT    PRED  RATE    OUT ')
TYPE 307, AO2EXT,AN2EXT,CO2EXT,H2OEXT,AON,ARATE,ARUN
307  FORMAT ( 4(F6.2,2X),F6.1,2X,F6.2,2X,F8.0,/)
C
C NEXT COMES THE CALCULATED VARIABLES
C
TYPE 308
308  FORMAT ( ' CALCULATED VARIABLES',/)
TYPE 309
309  FORMAT ( ' TOTCO2  CO2ER  TOTO2  OUR    KLA    RSPQUO
& VOLCO2')
TYPE 310, TOTCO2,CO2ER,TOTO2,OUR,KLA,RSPQUO,VOLCO2
310  FORMAT(1X,5(F6.1,3X),F8.2,3X,F6.1,/)
IF(DIFF.LT.0) THEN
ADIFF=0-DIFF
TYPE 314, ADIFF
314  FORMAT( ' UNDERFEEDING BY',F6.1,1X,' MINUTES')
ELSE
TYPE 315, DIFF
315  FORMAT( ' OVERFEEDING BY',F6.1,1X,' MINUTES')
END IF
IF(AIRFLG.EQ.1) THEN
TYPE 311
311  FORMAT( ' AIR FLOWRATE ENTERED MANUALLY',/)
END IF
IF(AIRFLG.EQ.0) THEN
TYPE 312
312  FORMAT( ' AIR FLOWRATE READ BY THE TCS UNIT',/)
END IF
C*****
C
C COMMENTING OUT THE CONTROL STRATEGY
C
C IF(ICOUN.EQ.1) THEN
C TYPE 91
C91  FORMAT(/,' NO CONTROL')
C END IF
C IF(ICOUN.EQ.2) THEN
C TYPE 92
C92  FORMAT(/,' AIR AND NITROGEN FLOWRATES SET')
C END IF
C IF(ICOUN.EQ.3) THEN

```

```
C      TYPE 93
C93    FORMAT(/,' AIR AND NITROGEN FLOWRATES CONTROLLED')
C      END IF
C*****
200    CONTINUE
      RETURN
      END
```

```

SUBROUTINE MKTIM
C..
C.. CALCULATES ELAPSED TIME SINCE INOCULATION.
C..
    INTEGER CYEAR,CMON,CDAY,CHR,CMIN,CSEC,CDAYNO
    INTEGER DAYS(12)
    BYTE CTIME(8),CDATE(9)
C..
    COMMON/TIMES/ ETIME
    COMMON/DATTIM/ CDATE,CTIME
    COMMON/CTIM/  CYEAR,CMON,CDAY,CHR,CMIN,CSEC
    COMMON/INCTIM/ IYEAR,IMON,IDAY,IHR,IMIN,ISEC
    DATA DAYS/0,31,59,90,120,151,181,212,243,273,304,334/
C..
    CALL TIME (CTIME)
    CALL DATE (CDATE)
    CALL IDATE (CMON,CDAY,CYEAR)
    DECODE (8,100,CTIME) CHR,CMIN,CSEC
100  FORMAT(I2,1X,I2,1X,I2)
C..
    IDAYNO=DAYS(IMON)+IDAY
    CDAYNO=DAYS(CMON)+CDAY
C..
    ETIME= (CYEAR-IYEAR)*8760. + (CDAYNO-IDAYNO)*24.
    &      + (CHR-IHR) + (CMIN-IMIN)/60. + (CSEC-ISEC)/3600.
C..
C    WRITE(5,200) IYEAR,IMON,IDAY,IHR,IMIN,ISEC,CDATE,CTIME,
C    &      CYEAR,CMON,CDAY,CHR,CMIN,CSEC,ETIME
C200  FORMAT(' ',6I4/9A1,8A1/6I4/F8.2)
    RETURN
    END

```

PROGRAM MKINIT

```

C
C THIS PROGRAM INITIALIZES RUN PARAMETERS USED THE DATA AQUISITION PROGRAMS
C MKMAIN,MKDATA, AND MKCHNG.
C
C MODIFIED MARCH, 1986 TO USE TCS BLOCK 3 FOR MONITORING D.O., PH, AND A
C GLUCOSE LOAD CELL. ALSO, A GLUCOSE CONTROL STRATEGY BASED ON CO2
C EVOLUTION IS NOW INCLUDED. SEE PROGRAMS JDCTRL,JDCTIN,JDFEED
C
C*****
C modified June, 1985, by Jim McMillan from Dave Robinson's DRINIT program
C*****
C modified August, 1986 by Max Kennedy from Jim McMillan's JDINIT programme
C
C 1, PROJECT: USE OF SOLID OXYGEN STORAGE MATERIALS
C Inclusion of Max's algorithm to control the oxygen content of gas
C sparged above the fermentaton liquid using air and nitrogen mixing
C by two control valves. This is to achieve an equality of partial
C pressures of oxygen in and above the fermentation liquid.
C This has been done at the expense of Jim's glucose control
C programme which has been deleted.
C
C 2, Setting of AIRFLG in the initialization programme instead
C of only being able to do this in MKCHNG
C
C GLOBAL COMMON AREAS:
C      Max =TCS101
C      Greg=TCS102
C      Dave=TCS104
C      Jim =TCS105
C
C GLOBAL COMMONS 64 BYTES
C      Integers = 2 bytes
C      Real no's = 4 bytes
C      Bytes    = as defined
C
C ADDED VARIABLES:
C      ICOUN = control strategy counter
C      ASET  = set value of air flowrate
C      ANSET = set value of nitrogen flowrate
C      ACON  = conversion factor for air flow (lpm/volt)
C      ANCON = conversion factor for nitrogen flow (lpm/volt)
C      MANA  = print control counter
C
C DELETED VARIABLES:
C      TOTCO2
C      AIRCON
C      GLULDO
C      GLUCON
C
C*****

```



```

C
  TYPE*, 'GIVE 6-CHAR. EXPERIMENT NO.'
  READ(5,10) BATCHN
10  FORMAT(6A1)
    DO 15 J=14,19
      PRMFIL(J)=BATCHN(J-13)
      DATFIL(J)=BATCHN(J-13)
      CLCFIL(J)=BATCHN(J-13)
      TCSFIL(J)=BATCHN(J-13)
      GLUFIL(J)=BATCHN(J-13)
15  TINFIL(J)=BATCHN(J-13)
C
  TLAST=0.0
  TOTCO2=0.0
  TOTO2=0.0
  APUMP=0.0
  VOLCO2=0.0
C
C
C INPUT OF VARIABLES TRANSFERRRED BY GLOBAL COMMON TSC101
  TYPE*, 'GIVE INOCULATION DATE AND TIME '
  TYPE*, 'MM/DD/YY HH:MM:SS '
  READ(5,25) IMON,IDAY,IYEAR,IHR,IMIN,ISEC
25  FORMAT(I2,1X,I2,1X,I2,1X,I2,1X,I2,1X,I2)
C
  OPEN(UNIT=1,TYPE='NEW',ACCESS='DIRECT',
&      NAME=PRMFIL,RECL=16)
  ILAST=0
  WRITE(1'1) ILAST,TLAST
  WRITE(1'2) IYEAR,IMON,IDAY,IHR,IMIN,ISEC
  CLOSE(UNIT=1)
C
  TYPE*, 'SCAN INTERVAL IN MINUTES?'
  ACCEPT*,SCNINT
  TYPE*, 'HOW MANY SCANS?'
  ACCEPT*,ISCAN
C
  TYPE*, 'MS CHANNEL FOR EXIT GAS? '
  ACCEPT*,MSEXCN
  TYPE*, 'MS CHANNEL FOR INLET GAS? '
  ACCEPT*,MSINCN
C
C
C
C SETTING AIRFLG TO ACCEPT MANUAL INPUT OF GAS FLOWRATE OR FROM THE TCS
  TYPE*, 'AIR FLOW SPARGED INTO THE BASE OF THE FERMENTER'
  TYPE*, 'DO YOU WANT'
555 TYPE*, '      0, AIR FLOWRATE READ FROM CONTROLLER'
  TYPE*, '      BY THE TCS UNIT'
  TYPE*, '      1, AIR FLOWRATE ENTERED MANUALLY'
  ACCEPT*,AIRFLG

```

```

IF (AIRFLG.LT.0.OR.AIRFLG.GT.1) THEN
TYPE*, ''
TYPE*, 'ENTER 0 OR 1'
TYPE*, ''
GOTO 555
END IF
556 IF (AIRFLG.EQ.1) THEN
TYPE*, 'ENTER AIR FLOWRATE (l/min) (CORRECTED TO 1 ATM!):'
ACCEPT*,AIRF
END IF
IF (AIRFLG.EQ.0) THEN
AIRF=0.0
END IF
C
TYPE*, 'CONVERSION FACTOR FOR D.O. OUTPUT'
TYPE*, 'USUALLY 1.0 (ATM O2/VOLT)?'
ACCEPT*,DO2CON
C
TYPE*, 'FERMENTER OPERATING PRESSURE'
TYPE*, 'USUALLY 14.7 (PSIA)?'
ACCEPT*,PFERM
C
TYPE*, 'INITIAL AQUEOUS VOLUME (L)?'
ACCEPT*,AQVOL
C
TYPE*, 'CELL CONC OF INOCULUM (g/l)?'
ACCEPT*,AINOC
C
TYPE*, 'INITIAL GLUCOSE CONC (g/l)?'
ACCEPT*,AGLUC
C
TYPE*, 'GLUCOSE SET POINT (g/l)?'
ACCEPT*,SGLU
C
TYPE*, 'GLUCOSE FEED STREAM CONC'
TYPE*, 'USUALLY 400 (g/l)?'
ACCEPT*,FGLU
C
TYPE*, 'SLOPE OF TOTAL CO2/CELL CONC CURVE'
TYPE*, 'USUALLY 34.4 (mmoles/g)?'
ACCEPT*,SLOPE
C
TYPE*, 'INTERCEPT OF TOTAL CO2/CELL CONC CURVE'
TYPE*, 'USUALLY 0.0 (mmoles)?'
ACCEPT*,AINTER
C
TYPE*, 'CELL PRODUCTION/GLUCOSE UTILIZATION'
TYPE*, 'USUALLY 0.4 (g/g)'
ACCEPT*,ACELL
C
C THIS CONTROL STRATEGY HAS NOW BEEN COMMENTED OUT

```



```

C
C SETTING THE CONTROL STRATEGY
C
C   TYPE*, 'GAS PHASE ABOVE THE FERMENTATION BROTH'
C   TYPE*, 'DO YOU WANT'
C300  TYPE*, '      1, NO CONTROL'
C   TYPE*, '      2, SET FLOWRATES OF AIR AND NITROGEN'
C   TYPE*, '      3, CONTROL OF AIR AND NITROGEN FLOWRATES'
C   TYPE*, '      BASED ON DO LEVEL IN THE BROTH'
C   ACCEPT*,ICOUN
C   IF (ICOUN.LT.1.OR.ICOUN.GT.3) THEN
C   TYPE*, ''
C   TYPE*, 'ENTER 1,2, OR 3'
C   TYPE*, ''
C   GOTO 300
C   END IF
C
C
C SETTING THE AIR AND NITROGEN FLOWRATES IF REQUIRED
C   IF (ICOUN.EQ.1) THEN
C   ASET=0.0
C   ANSET=0.0
C   END IF
C   IF (ICOUN.EQ.2) THEN
C   TYPE*, 'INPUT AIR FLOWRATE (lpm)'
C   ACCEPT*,ASET
C   TYPE*, 'INPUT NITROGEN FLOWRATE (lpm)'
C   ACCEPT*,ANSET
C   END IF
C
C
C CONVERSION FACTORS FOR FLOWRATES (LPM TO VOLTS)
C   TYPE*, 'ABOVE THE FERMENTATION BROTH:'
C   TYPE*, 'ENTER CONVERSION FACTOR FOR AIR FLOWRATE (lpm/volt)'
C   ACCEPT*,ACON
C   TYPE*, 'ABOVE THE FERMENTATION BROTH:'
C   TYPE*, 'ENTER CONVERSION FACTOR FOR NITROGEN FLOWRATE (lpm/volt)'
C   ACCEPT*,ANCON
C
C
C END OF INPUT OF DATA
C   TYPE*, 'THANK YOU'
C
C
C OPENING OUTPUT FILE FOR INITIALIZATION DATA
C   OPEN (UNIT=2,TYPE='NEW',ACCESS='SEQUENTIAL',NAME=TINFIL)
C   WRITE(2,500)
500  FORMAT(' INITIALIZATION FILE',/,/,/,/,/,/)
C
C
C WRITING TO SCREEN TO CHECK INPUT DATA AND TO A FILE FOR A PERMANENT

```

```

C RECORD
  MANA=5
  DO 900 JJ=1,2
  WRITE(MANA,90) BATCHN
90  FORMAT( /,' THE INITIAL CONDITIONS FOR RUN ',6A1,' ARE:',/)
C
  WRITE(MANA,200) IDAY,IMON,IYEAR,IHR,IMIN,ISEC
200  FORMAT(' INOCULATION TIME',/, ' DAY  =' ,I2,4X,' MONTH=' ,I2,
&      4X,' YEAR  =' ,I2,/, ' HOUR  =' ,I2,4X,' MIN   =' ,I2,
&      4X,' SEC   =' ,I2,/)
  WRITE(MANA,201)
201  FORMAT(' SCAN  NUMBER  EXIT  INLET  VOL  AIR')
  WRITE(MANA,202)
202  FORMAT(' INTERVAL  OF  GAS  GAS  H2O  FLOW')
  WRITE(MANA,203)
203  FORMAT(' (min)  SCANS  CHANNEL  CHANNEL  (l)  (l/min)')
C
  WRITE(MANA,100) SCNINT,ISCAN,MSEXCN,MSINCN,AQVOL,AIRF
C
100  FORMAT(1X,F5.1,4X,I5,3X,I5,3X,I5,4X,F5.2,2X,F5.2,2X,F5.2)
  WRITE(MANA,301)
301  FORMAT(/)
C
  WRITE(MANA,204)
204  FORMAT(' DO2CON PFERM  INIT  INOC  SLOPE  INTER  CELL
&  GLUC  GLUC')
  WRITE(MANA,205)
205  FORMAT(' (atm/V) (psia) GLUC  CELL  CO2  CO2  /GLUC
&  SET PT FEED')
  WRITE(MANA,214)
214  FORMAT('          (g/l) (g/l) (mM/g)  (mM)  (g/g)
&  (g/l) (g/l)')
C
  WRITE(MANA,110) DO2CON,PFERM,AGLUC,AINOC,SLOPE,AINTER,ACELL,
&  SGLU,FGLU
110  FORMAT(1X,F5.1,3X,F5.1,2X,F5.1,2X,F5.1,2X,F5.2,2X,F5.2,2X,F5.2
&  ,2X,F5.2,2X,F5.1)
C
C
C CONTROL STATUS PRINTOUTS HAVE BEEN COMMENTED OUT
C
C
C  WRITE(MANA,206)
C206  FORMAT(/,' CONTROL STATUS :ABOVE THE FERMENTATION BROTH')
C  IF (ICOUN.EQ.1) THEN
C  WRITE(MANA,207)
C207  FORMAT(' NO CONTROL')
C  END IF
C  IF (ICOUN.EQ.2) THEN
C  WRITE(MANA,208)
C208  FORMAT(' AIR AND NITROGEN FLOWRATES SET')

```

```

C    WRITE(MANA,230) ASET,ANSET
C230  FORMAT(' AIR FLOWRATE      = ',F6.2,' lpm',
C    &      /,' NITROGEN FLOWRATE = ',F6.2,' lpm')
C    END IF
C    IF (ICOUN.EQ.3) THEN
C    WRITE(MANA,209)
C209  FORMAT(' AIR AND NITROGEN FLOWRATES CONTROLLED')
C    END IF
C
C
C    WRITE(MANA,211)
211  FORMAT(/,' STATUS: AIR ENTERING THE FERMENTER BASE')
C    IF (AIRFLG.EQ.0) THEN
C    WRITE(MANA,212)
212  FORMAT(' AIR FLOWRATE READ BY THE TCS UNIT')
C    END IF
C    IF (AIRFLG.EQ.1) THEN
C    WRITE(MANA,213)
213  FORMAT(' AIR FLOWRATE ENTERED MANUALLY')
C    END IF
900  MANA=2
C
C
C CLOSING INITIALIZATION FILE
C    CLOSE (UNIT=2)
C    STOP
C    END

```

PROGRAM MKCHNG

```

C   ALLOWS SYSTEM & SCANNING PARAMETERS TO BE CHANGED
C   DURING A RUN
C
C   MODIFIED TO USE TCS DATA MARCH, 1986
C
C*****
C   Modified June,1985, by Jim McMillan from Dave Robinson's DRCHNG
C   program
C*****
C   Modified August,1986,by Max Kennedy from Jim McMillan's JDCHNG
C
C   1, Modified to change variables,ICOUN,ASET,ANSET,ACON,ANCON for
C   solid oxygen storage materials project.
C
C   2, Modified to change mass spec channels during fermentation.
C
C   3, Modified to print changed variables to a print file
C*****
C   Modified on 1/20/88 by Max Kennedy
C   new programme for protease experiments
C*****
C   Modified on 2/6/89 by Max Kennedy
C   glucose control variables added
C*****
C
C   SEE SUBROUTINES MKDATA AND MKINIT FOR AN EXPLANATION OF VARIABLES
C
C*****
C       BYTE CHGFIL(24),BATCHN(6)
C       INTEGER AIRFLG
C       REAL NH3INL,NH3EXT,KLA
C
C       COMMON/TCS101/ BATCHN,SCNINT,ISCAN,MSEXCN,MSINCN,AQVOL,
&           AIRF,AIRFLG,IREC,TLAST,PFERM,
&           DO2CON,AINOC,AGLUC,SLOPE,AINTER,ACELL,APUMP
C
C       COMMON/TCS103/ VOLCO2,TOTCO2,TOTO2,SGLU,FGLU
C
C   PREVENT OUTPUT OR CONVERSION ERRORS FROM STOPPING THE PROGRAM
CALL ERRSET (63,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (70,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (71,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (72,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (73,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (74,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (75,.TRUE.,.FALSE.,,.FALSE.,500)
CALL ERRSET (83,.TRUE.,.FALSE.,,.FALSE.,500)
C
C

```

A-26

```

C504  FORMAT(' AIR FLOWRATE      = ',F6.2,' lpm',
C    &      /,' NITROGEN FLOWRATE = ',F6.2,' lpm')
C    END IF
C    IF(ICOUN.EQ.3) THEN
C    WRITE(MANA,505)
C505  FORMAT(' AIR AND NITROGEN FLOWRATES CONTROLLED')
C    END IF
C
C
C    WRITE(MANA,506)
506  FORMAT(/,' STATUS: AIR ENTERING THE FERMENTER BASE')
C    IF (AIRFLG.EQ.0) THEN
C    WRITE(MANA,507)
507  FORMAT(' AIR FLOWRATE READ BY THE TCS UNIT',/,/)
C    END IF
C    IF (AIRFLG.EQ.1) THEN
C    WRITE(MANA,508)
508  FORMAT(' AIR FLOWRATE ENTERED MANUALLY',/,/)
C    END IF
C
C
C THIS STATEMENT JUMPS YOU TO THE END IF THE .CHG FILE HAS BEEN WRITTEN
C    IF(TRIP.EQ.1) GOTO 400
C    TYPE*, ' '
C    WRITE(5,509)
509  FORMAT(' TO CHANGE A VARIABLE ENTER ITS NUMBER > ', $)
C    READ (5,4) ICHGIT
4    FORMAT(I6)
C    IF(ICHGIT.EQ.99) ICHGIT=18
C    IF((ICHGIT.LT.1) .OR. (ICHGIT.GT.18)) GOTO 2
C    GOTO (110,120,130,140,200,160,170,180,190
2      ,310,320,330,340,350,360,380,390,300) ICHGIT
C
110    WRITE(5,101) ISCAN
C    READ (5,4) ISCAN
C    GOTO 2
120    WRITE(5,102) SCNINT
C    READ (5,100) SCNINT
C    GOTO 2
130    WRITE(5,103) AIRF
C    READ (5,100) AIRF
C    GOTO 2
140    WRITE(5,104) AQVOL
C    READ (5,100) AQVOL
C    GOTO 2
160    WRITE(5,106) PFERM
C    READ (5,100) PFERM
C    GOTO 2
170    WRITE(5,107) DO2CON
C    READ (5,100) DO2CON
C    GOTO 2

```

```

180      WRITE(5,108) MSINCN
        READ (5,4) MSINCN
        GOTO 2
190      WRITE(5,109) MSEXCN
        READ (5,4) MSEXCN
        GOTO 2
200      WRITE(5,111) AIRFLG
        READ (5,4) AIRFLG
        IF (AIRFLG.LT.0.OR.AIRFLG.GT.1) THEN
          TYPE*, ' '
          TYPE*, ' ENTER 0 OR 1'
          TYPE*, ' '
          GOTO 200
        END IF
        GOTO 2
310      WRITE(5,117) AINOC
        READ (5,100) AINOC
        GOTO 2
320      WRITE(5,118) AGLUC
        READ (5,100) AGLUC
        GOTO 2
330      WRITE(5,119) SLOPE
        READ (5,100) SLOPE
        GOTO 2
340      WRITE(5,122) AINTER
        READ (5,100) AINTER
        GOTO 2
350      WRITE(5,121) ACELL
        READ (5,100) ACELL
        GOTO 2
360      WRITE(5,123) APUMP
        READ (5,100) APUMP
        GOTO 2
380      WRITE(5,124) SGLU
        READ (5,100) SGLU
        GOTO 2
390      WRITE(5,125) FGLU
        READ (5,100) FGLU
        GOTO 2
C
C COMMENTING OUT CONTROL SECTION
C
C210      WRITE(5,112) ICOUN
C          READ (5,4) ICOUN
C          IF (ICOUN.LT.1.OR.ICOUN.GT.3) THEN
C            TYPE*, ' '
C            TYPE*, ' ENTER 1,2, OR 3'
C            TYPE*, ' '
C            GOTO 210
C          END IF
C          IF (ICOUN.EQ.1) THEN

```

```

C    ASET=0.0
C    ANSET=0.0
C    END IF
C    GOTO 2
C220    WRITE(5,113) ASET
C    READ (5,100) ASET
C    GOTO 2
C230    WRITE(5,114) ANSET
C    READ (5,100) ANSET
C    GOTO 2
C240    WRITE(5,115) ACON
C    READ (5,100) ACON
C    GOTO 2
C250    WRITE(5,116) ANCON
C    READ (5,100) ANCON
C    GOTO 2
C
C*****
C
100    FORMAT(F8.3)
101    FORMAT('      ISCAN = ',I6,'      NEW VALUE = ')
102    FORMAT('      SCNINT = ',F6.3,'      NEW VALUE = ')
103    FORMAT('      AIRF = ',F6.3,'      NEW VALUE = ')
104    FORMAT('      AQVOL = ',F6.3,'      NEW VALUE = ')
106    FORMAT('      PFERM = ',F6.3,'      NEW VALUE = ')
107    FORMAT('      DO2CON = ',F6.3,'      NEW VALUE = ')
108    FORMAT('      MSINCN = ',I6,'      NEW VALUE = ')
109    FORMAT('      MSEXCN = ',I6,'      NEW VALUE = ')
111    FORMAT('      AIRFLG = ',I6,'      NEW VALUE = ')
C
C COMMENTING OUT CONTROL VARIABLES
C
C112    FORMAT('      ICOUN = ',I6,'      NEW VALUE = ')
C113    FORMAT('      ASET = ',F6.3,'      NEW VALUE = ')
C114    FORMAT('      ANSET = ',F6.3,'      NEW VALUE = ')
C115    FORMAT('      ACON = ',F6.3,'      NEW VALUE = ')
C116    FORMAT('      ANCON = ',F6.3,'      NEW VALUE = ')
C
117    FORMAT('      AINOC = ',F6.3,'      NEW VALUE = ')
118    FORMAT('      AGLUC = ',F6.3,'      NEW VALUE = ')
119    FORMAT('      SLOPE = ',F6.3,'      NEW VALUE = ')
122    FORMAT('      AINTER = ',F6.3,'      NEW VALUE = ')
121    FORMAT('      ACELL = ',F6.3,'      NEW VALUE = ')
123    FORMAT('      APUMP = ',F6.1,'      NEW VALUE = ')
124    FORMAT('      SGLU = ',F6.3,'      NEW VALUE = ')
125    FORMAT('      FGLU = ',F6.1,'      NEW VALUE = ')
C
251    FORMAT(' LAST READING TAKEN AT : ',F6.2,/)
C
C REPEAT LOOP: ARE THERE ANY OTHER VARIABLES TO CHANGE?
C

```



```
300  CONTINUE
C
C
C SET THE UNIT NUMBER TO 1 TO WRITE TO THE OUTPUT FILE
  MANA=1
  TRIP=1.0
  GOTO 444
400  CONTINUE
  CLOSE (UNIT=1)
  STOP
  END
```

PROGRAM MKPRNT

```

C PROGRAM TO PRINT DATA GENERATED DURING RUNS
C
C*****
C
C Modified April,1986, by Jim McMillan to include new files:
C   TCSFIL -- a new data file for MKDATA
C
C Modified June,1985, by Jim McMillan from Dave Robinson's DRPRNT program
C
C*****
C
C Modified August,1985 by Max Kennedy from Jim McMillan's JDPRNT programme
C
C   1 Print variables from air and nitrogen flow control project added
C
C   2 Print variables from Jim's glucose control strategy deleted
C
C*****
C
C SEE SUBROUTINE MKDATA FOR AN EXPLANATION OF VARIABLES
C
C*****
C
C ORGANIZATION OF THE DATA FILES:
C
C   There are 6 data files for each run: The initialization file
C (TINFIL),the change file (CHGFIL), the parameter file (PRMFIL),
C calculation file (CLCFIL), data file (DATFIL), and the TCS file
C (TCSFIL) which includes KLA and driving forces. The parameter
C file contains only two pieces of information which are continually
C being updated: the number of scans recorded, IREC, and the elapsed
C time, ETIME. The data file contains, in addition to the number of
C scans and the elapsed time, the gas composition data for both the
C inlet and outlet gas streams. Percent nitrogen, oxygen, carbon dioxide,
C water vapor, ethanol, hydrogen, and ammonia are all recorded. Finally,
C other relevant parameters, both input and calculated from off-gas data,
C are recorded in the calculation file. As well as IREC and ETIME, the
C actual date and time are also recorded in the calculation file.
C The TCSFIL is merely an extension of the calculation file.
C*****
C
C UPDATED IN JAN 88 BY MAX KENNEDY FOR THE PROTEASE PROJECT
C MAIN CHANGES ARE THE PRINTING OUT OF MORE DATA ESPECIALLY THE
C MASS SPEC DATA.
C
C*****
C   BYTE DATFIL(24),PRMFIL(24), CLCFIL(24),USERNO,TCSFIL(24)
C   BYTE GLUFIL(24)
C   INTEGER CYEAR,CMON,CDAY,CHR,CMIN,CSEC,AIRFLG

```

Address	Disassembly	Comment
00000000	REAL NH3INL,NH3EXT,KLA	
00000001	COMMON/PRTCOM/IPRT,ILAST,ETLAST,DATFIL,MANA	
00000002		
00000003		
00000004		
00000005	READ(5,5) (DATFIL(J),J=14,19)	
00000006	FORMAT(6A1)	
00000007	DO 7 J =14,19	
00000008	CLCFIL(J)=DATFIL(J)	
00000009	TCSFIL(J)=DATFIL(J)	
0000000A	GLUFIL(J)=DATFIL(J)	
0000000B	PRMFIL(J)=DATFIL(J)	
0000000C	TYPE*,PLEASE enter 6-CHARACTER Experiment Name'	
0000000D		
0000000E		
0000000F		
00000010		
00000011		
00000012		
00000013		
00000014		
00000015		
00000016		
00000017		
00000018		
00000019		
0000001A		
0000001B		
0000001C		
0000001D		
0000001E		
0000001F		
00000020		
00000021		
00000022		
00000023		
00000024		
00000025		
00000026		
00000027		
00000028		
00000029		
0000002A		
0000002B		
0000002C		
0000002D		
0000002E		
0000002F		
00000030		
00000031		
00000032		
00000033		
00000034		
00000035		
00000036		
00000037		
00000038		
00000039		
0000003A		
0000003B		
0000003C		
0000003D		
0000003E		
0000003F		
00000040		
00000041		
00000042		
00000043		
00000044		
00000045		
00000046		
00000047		
00000048		
00000049		
0000004A		
0000004B		
0000004C		
0000004D		
0000004E		
0000004F		
00000050		
00000051		
00000052		
00000053		
00000054		
00000055		
00000056		
00000057		
00000058		
00000059		
0000005A		
0000005B		
0000005C		
0000005D		
0000005E		
0000005F		
00000060		
00000061		
00000062		
00000063		
00000064		
00000065		
00000066		
00000067		
00000068		
00000069		
0000006A		
0000006B		
0000006C		
0000006D		
0000006E		
0000006F		
00000070		
00000071		
00000072		
00000073		
00000074		
00000075		
00000076		
00000077		
00000078		
00000079		
0000007A		
0000007B		
0000007C		
0000007D		
0000007E		

```

8   FORMAT(/' NO. OF RECORDS =',I5/' TOTAL FERM. TIME =',F8.2//)

C
    WRITE(5,18)
18   FORMAT(/' GIVE INITIAL PRINT TIME, FINAL PRINT TIME,
    & PRINT INTERVAL (HR):')
    READ(5,*) TPI,TPF,DTP
    WRITE(5,20)
20   FORMAT(/' VT105 = 5, DEC IV = 6, LA50 = 7, WHICH ONE?')
    READ(5,*) IPRT
    IF(IPRT.EQ.7) CALL CHG132(IPRT)
90   CONTINUE
C
C THIS LOOP FIRST PRINTS OUT THE CALCULATED DATA THEN PRINTS THE
C MASS SPEC DATA
C
    DO 500 MANA=1,3
    CALL HEDPRT
    PTLAST=0.0
    JHCNT=0
    DO 200 J=1,ILAST
C*****
C COMMENTED OUT: READ STATEMENTS FOR JMC001 THRU JMC004, AND FOR
C   JMFC01 AND JMFC02
C
C   READ(2'J) IREC,ETIME,AN2EXT,AO2EXT,CO2EXT,H2OEXT,ETHEXT,
C   &   AH2EXT,NH3EXT,AN2INL,AO2INL,CO2INL,H2OINL,ETHINL,
C   &   AH2INL,NH3INL
C
C   READ(3'J) IREC,CYEAR,CMON,CDAY,CHR,CMIN,CSEC,ETIME,AQVOL,
C   &   FCVOL,AIRF,OUR,CO2ER,TOTO2,TOTCO2,RSPQUO
C*****
C
C   READ(2'J) IREC,ETIME,AN2EXT,AO2EXT,CO2EXT,H2OEXT,
C   &   ETHEXT,AH2EXT,NH3EXT,AN2INL,AO2INL,CO2INL,H2OINL,
C   &   ETHINL,AH2INL,NH3INL
C
C   READ(3'J) IREC,CYEAR,CMON,CDAY,CHR,CMIN,CSEC,
C   &   ETIME,AQVOL,AIRF,OUR,CO2ER,TOTO2,TOTCO2,
C   &   RSPQUO
C
C   READ(4'J) IREC,ETIME,PFERM,DO2CON,PLIQO2,PGASO2,KLA,
C   &   RFLOW1,CELL,AON,ZGLU,ARATE,ARUN,AUSED,
C   &   SCNINT,AIRFLG
C
C   READ(8'J) AINOC,ACELL,SLOPE,AINTER,AGLUC,APUMP,DIFF,
C   &   SGLU,VOLCO2,FGLU
C
C CHECK IF THE DATA BEING READ SHOULD BE PRINTED
C
    TLAST=ETIME

```

```

IF(ETIME.LT.TPI) GO TO 200
IF(ETIME.GT.TPF) GO TO 300
IF(ETIME.LT.(PTLAST+DTP)) GO TO 200

C
C
C
JHCNT=JHCNT+1
IF (JHCNT.GE.51) THEN
  JHCNT=0
  CALL HEDPRT
ENDIF

C*****
C WRITE STATEMENT FOR JMC001-JMC004, JMF001-JMF002
C
C   WRITE(IPRT,195) J,CMON,CDAY,CYEAR,CHR,CMIN,ETIME,
C   &  AN2EXT,AO2EXT,CO2EXT,H2OEXT,AH2EXT,NH3EXT,AIRF,
C   &  AQVOL,FCVOL,OUR,TOTO2,RSPQUO
C
C195  FORMAT(' ',I4,I4,'/',I2,'/',I2,2X,I2,':',I2,F8.2,
C   &      1X,8(F7.2,1X),F7.3,1X,
C   &      F7.2,1X,2(F7.1,1X))
C*****
C
C DECIDES WHICH DATA TO PRINT OUT, MASS SPEC OR NON-MASS SPEC DATA
C
  IF (MANA.EQ.2) THEN
    GOTO 511
  END IF
  IF (MANA.EQ.3) THEN
    GOTO 512
  END IF
  WRITE(IPRT,195) J,CMON,CDAY,CYEAR,CHR,CMIN,ETIME,SCNINT,
  &      PLIQO2,AIRFLG,AIRF,AQVOL,PFERM,
  &      OUR,TOTO2,CO2ER,TOTCO2,KLA,RSPQUO
C
195  FORMAT(' ',I4,I4,'/',I2,'/',I2,2X,I2,':',I2,F8.3,1X,
  &      F7.2,3X,F7.3,2X,I2,2X,4(F7.2,2X),F7.2,2X,
  &      F7.2,2X,F7.2,2X,F7.2,2X,F7.2)
  GOTO 510
C
C PRINTS OUT THE MASS SPEC DATA
C
511  WRITE(IPRT,196) J,ETIME,AO2INL,AN2INL,CO2INL,H2OINL,
  &      ETHINL,AH2INL,NH3INL,AO2EXT,AN2EXT,
  &      CO2EXT,H2OEXT,ETHEXT,AH2EXT,NH3EXT
C
196  FORMAT(' ',I4,1X,F8.3,1X,14(F7.3,1X))
  &
  &
  GOTO 510
512  WRITE(IPRT,197) J,ETIME,AINOC,ACELL,CELL,SLOPE,AINTER,APUMP,

```

```

&          AON,AGLUC,ZGLU,AUSED,ARATE,ARUN,DIFF,SGLU,FGLU,
&          VOLCO2
197  FORMAT(' ',I4,1X,6(F6.2,1X),2(F6.1,1X),3(F6.2,1X),F7.3,1X,
&          F8.0,1X,F6.1,1X,F5.1,1X,
&          F6.1,1X,F6.1)
510  CONTINUE
C
      PTLAST=ETIME
C
C
200  CONTINUE
300  CONTINUE
500  CONTINUE
C
      IF(IPRT.EQ.7) CALL CHG080(IPRT)
C
      CLOSE(UNIT=1)
      CLOSE(UNIT=2)
      CLOSE(UNIT=3)
      CLOSE(UNIT=4)
      CLOSE(UNIT=8)
C
      STOP
      END

```

C*****SUBROUTINE FOR TYPING UP HEADING*****

```

      SUBROUTINE HEDPRT
      BYTE DATFIL(24)
      COMMON/PRTCOM/IPRT,ILAST,ETLAST,DATFIL,MANA

      WRITE(IPRT,30) (DATFIL(J),J=14,19),ILAST,ETLAST
30    FORMAT('1',/,3X,' EXPT NO. ',6A1,25X,' NO. OF RECORDS =',I4
&          ,10X,' TOTAL FERM. TIME =',F7.2
&          ,/,', ',132('-'),/,',+',132('_'),/)
C
C DECIDES IF ITS TO PRINT THE MASS SPEC OR NON-MASS SPEC DATA
C
      IF (MANA.EQ.2) THEN
        GOTO 501
      END IF
      IF (MANA.EQ.3) THEN
        GOTO 503
      END IF
C
C THE HEADING FOR THE NON-MASS SPEC DATA
C
      WRITE(IPRT,95)
95    FORMAT(' REC',22X,'ELAP.',3X,'SCAN',6X,'D.O.',
&          3X,'AIR',4X,'AIR',
&          4X,'AQUEOUS',2X,'PRESS',5X,'OUR',5X,'TOTAL',4X,'CO2ER',

```

```

&      4X,'TOTAL',6X,'KLA',5X,'RESP',/,
&      ' ',NO.',5X,'DATE',5X,'TIME',4X,'TIME',
&      4X,'INT',6X,'(atm)',2X,'FLAG',3X,'FLOW',
&      4X,'VOL(l)',3X,'(psia)',
&      4X,' ',5X,'O2',4X,' ',10X,'CO2',
&      4X,' ',9X,'QUO',/,
&      132(' '),/, '+ ',132(' ')./)
      GOTO 502
C
C THE HEADING FOR THE MASS SPEC DATA
C
501  WRITE(IPRT,96)
96   FORMAT(' REC',5X,'ELAP.',5X,'O2',6X,'N2',
&          5X,'CO2',5X,'H2O',
&          5X,'ETOH',5X,'H2',5X,'NH3',6X,'O2',6X,'N2',
&          5X,'CO2',5X,'H2O',5X,'ETOH',5X,'H2',5X,'NH3',/,
&          ' ',NO.',5X,'TIME',5X,'INL',5X,'INL',
&          5X,'INL',5X,'INL',5X,'INL',5X,'INL',
&          5X,'INL',5X,'OUT',
&          5X,'OUT',5X,'OUT',5X,'OUT',5X,'OUT',
&          5X,'OUT',5X,'OUT',/,
&          132(' '),/, '+ ',132(' ')./)
      GOTO 502
C
C THE HEADING FOR THE GLUCOSE CONTROL DATA
C
503  WRITE(IPRT,97)
97   FORMAT(' REC',4X,'ELAP.',2X,'INOC',3X,'X/S ',
&          2X,'CELL',2X,'SLOPE',
&          2X,'INTER',3X,'PUMP',3X,'PUMP',3X,'GLUC',3X,'GLUC',
&          3X,'GLUC',3X,'GROWTH',3X,'RUN',4X,'PUMP',3X,'GLUC',
&          2X,'GLUC',4X,'VOL',/,
&          ' ',NO.',4X,'TIME',3X,'CONC',3X,' ',
&          3X,'CONC',3X,'CO2',4X,'CO2',3X,'ACTUAL',
&          2X,'PRED',3X,'INIT',
&          3X,'CONC',3X,'USED',4X,'RATE',4X,'OUT',4X,'DIFF',
&          3X,'SET',3X,'FEED',4X,'CO2',/,
&          132(' '),/, '+ ',132(' ')./)
502  CONTINUE
      RETURN
      END

```

PROGRAM MKPLOT

```

C FURTHER MODIFIED (WITH SOME OLD TEXT DELETED) 3/31/86 BY JIM MCMILLAN
C
C MODIFIED 6/15/85 TO READ [305,155] FILES BY JIM MCMILLAN*****
C MODIFIED 8/1/86 TO READ [314,45] FILES BY MAX KENNEDY
C MODIFIED 8/08/85 BY DAVE ROBINSON TO READ DATA FILES CORRESPONDING
C TO DRDAT2 AND CHDATA FILE STRUCTURE
C
C*****
C
C MODIFIED 8/20/86 BY M.J. Kennedy
C
C 1 Addition of variables from air and nitrogen valve control
C
C 2 Deletion of variables from Jim's glucose control strategy
C
C*****
C
C.. PROGRAM TO PLOT EXIT GAS COMPOSITION AND TOTAL GAS PRODUCED.
C.. MODIFICATION TO PRINT ON TT5: 11/12/84
C..
    BYTE BATCHN(6),DATFIL(24),PRMFIL(24),CLCFIL(24),TCSFIL(24)
    BYTE GLUFIL(24)
    BYTE XTITLE(21),ATITLE(21),BTITLE(21)
    BYTE GASNAM(4,6)
    INTEGER CYEAR,CMON,CDAY,CHR,CMIN,CSEC
    INTEGER IYEAR,IMON,IDAY,IHR,IMIN,ISEC,ISCR
    INTEGER AIRFLG
    REAL VALUE(30)
    REAL YAAXIS(2,6),YBAXIS(2,6)
    REAL KLA,AQVOL,FCVOL,NH3EXT,PLIQO2,PGASO2,RFLOW1,PFERM
    REAL AIRF,OUR,CO2ER,GLUFED
C
C EQUIVALENCE STATEMENTS MODIFIED 3/31/86
    EQUIVALENCE (VALUE(1),ETIME)
    EQUIVALENCE (VALUE(2),AN2INL)
    EQUIVALENCE (VALUE(3),AO2INL)
    EQUIVALENCE (VALUE(4),CO2INL)
    EQUIVALENCE (VALUE(5),H2OINL)
    EQUIVALENCE (VALUE(6),CELL)
    EQUIVALENCE (VALUE(7),NH3INL)
    EQUIVALENCE (VALUE(8),APUMP)
    EQUIVALENCE (VALUE(9),AN2EXT)
    EQUIVALENCE (VALUE(10),AO2EXT)
    EQUIVALENCE (VALUE(11),CO2EXT)
    EQUIVALENCE (VALUE(12),H2OEXT)
    EQUIVALENCE (VALUE(13),AON)
    EQUIVALENCE (VALUE(14),NH3EXT)
    EQUIVALENCE (VALUE(15),ZGLU)
    EQUIVALENCE (VALUE(16),VOLCO2)

```



```

EQUIVALENCE (VALUE(17),SGLU)
EQUIVALENCE (VALUE(18),AUSED)
EQUIVALENCE (VALUE(19),AIRF)
EQUIVALENCE (VALUE(20),AQVOL)
EQUIVALENCE (VALUE(21),ARATE)
EQUIVALENCE (VALUE(22),OUR)
EQUIVALENCE (VALUE(23),TOTO2)
EQUIVALENCE (VALUE(24),CO2ER)
EQUIVALENCE (VALUE(25),TOTCO2)
EQUIVALENCE (VALUE(26),KLA)
EQUIVALENCE (VALUE(27),RSPQUO)
EQUIVALENCE (VALUE(28),DIFF)
EQUIVALENCE (VALUE(29),ARUN)
C
C
C
COMMON/BTHDTA/ BATCHN,IYEAR,IMON,IDAY,ILBL
DIMENSION X(500),YA(500),YB(500)
COMMON/TITLES/ XTITLE,ATITLE,BTITLE
C..
DATA ILBL/1/
C..
C MODIFIED HERE 3/31/86 TO READ MKDATA FILES
C*****
DATA PRMFIL /'D','L','1',' ',' ',' ',' ','3','1','4',' ',' ','0','4','5',' ','
& 'X','X','X','X','X','X',' ','P','R','M',0/
DATA DATFIL /'D','L','1',' ',' ',' ',' ','3','1','4',' ',' ','0','4','5',' ','
& 'X','X','X','X','X','X',' ','D','T','M',0/
DATA CLCFIL /'D','L','1',' ',' ',' ',' ','3','1','4',' ',' ','0','4','5',' ','
& 'X','X','X','X','X','X',' ','D','T','C',0/
DATA TCSFIL /'D','L','1',' ',' ',' ',' ','3','1','4',' ',' ','0','4','5',' ','
& 'X','X','X','X','X','X',' ','T','C','S',0/
DATA GLUFIL /'D','L','1',' ',' ',' ',' ','3','1','4',' ',' ','0','4','5',' ','
& 'X','X','X','X','X','X',' ','G','L','U',0/
C*****
C
WRITE(5,2)
2 FORMAT(/' GIVE 6-CHAR. EXPERIMENT NO.: ',)
READ(5,5) BATCHN
5 FORMAT(6A1)
DO 7 J= 14,19
DATFIL(J) = BATCHN(J-13)
CLCFIL(J) = BATCHN(J-13)
TCSFIL(J) = BATCHN(J-13)
GLUFIL(J) = BATCHN(J-13)
7 PRMFIL(J) = BATCHN(J-13)
C
OPEN(UNIT = 1,TYPE = 'OLD',ACCESS = 'DIRECT',SHARED,READONLY,
& NAME = PRMFIL,RECL = 16)
READ(1'1) ILAST,ETLAST
READ(1'2) IYEAR,IMON,IDAY,IHR,IMIN,ISEC

```

```

C
      CLOSE(UNIT=1)
      WRITE(5,8) ILAST,ETLAST
      FORMAT(/'NO. OF RECORDS =',I5/'TOTAL FERM. TIME =',F8.2//)
      C
      WRITE(5,15)
      FORMAT(/'SPECIFY DATA BY ENTERING THE APPROPRIATE NUMBER: ',/
      &
      'ELAPSED TIME = 1',//;'*** GAS DATA ***',//,
      &
      'N2 INL = 2',5X,'O2 INL = 3',5X,
      &
      'CO2 INL = 4',//,
      &
      'H2O INL = 5',5X,'CELL CONC = 6',5X,
      &
      'NH3 INL = 7',//,
      &
      'PUMP ACT = 8',5X,'N2 OUT = 9',5X,
      &
      'O2 OUT = 10',//,
      &
      'CO2 OUT = 11',5X,'H2O OUT = 12',5X,
      &
      'PUMP PRED = 13',//,
      &
      'NH3 OUT = 14',5X,'GLUC CONC = 15',5X,
      &
      'VOLCO2 = 16',//,
      &
      'GL SET P = 17',5X,'GLUC USED = 18',5X,
      &
      'AIRFLOW = 19',//,
      &
      'AQ VOL = 20',5X,'GROW RATE = 21',//,
      &
      'OUR = 22',5X,'TOTOT2 = 23',5X,
      &
      'COZER = 24',//,
      &
      'TOTCO2 = 25',5X,'KLA = 26',5X,
      &
      'RSPQO = 27',//,
      &
      'PUMP DIF = 28',5X,'RUN OUT = 29',//,
      &
      'INDICATE YA,YB,X: ',/,$)
      READ(5,*) IYA, IYB, IX
      C..
      WRITE(5,40)
      FORMAT(/'GIVE MIN. AND MAX. VALUE FOR LEFT Y-AXIS GRAPH: ',/,$)
      READ(5,*) YAMIN,YAMAX
      WRITE(5,45)
      FORMAT(/'GIVE MIN. AND MAX. VALUE FOR RT. Y-AXIS GRAPH: ',/,$)
      READ(5,*) YBMIN,YBMAX
      WRITE(5,50)
      FORMAT(/'GIVE MIN. AND MAX. VALUE FOR X GRAPH: ',/,$)
      READ(5,*) XMIN,XMAX
      C..ALLOW ONE TO SKIP ENTERING AXIS TITLES
      WRITE(5,120)
      FORMAT(/'DO YOU WISH TO ENTER AXIS TITLES?[YES=1, NO=0]:',/,$)
      READ(5,*) IAXIS
      IF(IAXIS.EQ.0) GOTO 180
      C..ALLOW A DEFAULT X AXIS TITLE
      WRITE(5,130)
      FORMAT(/'DEFAULT X-AXIS TITLE?, [YES=1, NO=0]:',/,$)
      READ(5,*) IXXAXIS
      IF(IXXAXIS.EQ.1) THEN
        DATA XTITLE /'E','L','A','P','S','E','D',/,
        'T','I','M','E',/,/,'(', 'H','O','U','R','S',/,'/',
        GOTO 158
      &

```

```

ENDIF
WRITE(5,150)
150  FORMAT(/' ENTER X-AXIS TITLE (UPTO 20 CHAR.):'/
1      ' 0      1      2'/
1      ' 12345678901234567890' /)
READ(5,155) (XTITLE(I),I=1,20)
155  FORMAT(40A1)
158  CONTINUE
WRITE(5,160)
160  FORMAT(/' ENTER LEFT-HAND Y-AXIS TITLE (UPTO 20 CHAR.):'/
1      ' 0      1      2'/
1      ' 12345678901234567890' /)
READ(5,165) (ATITLE(I),I=1,20)
165  FORMAT(20A1)
WRITE(5,170)
170  FORMAT(/' ENTER RIGHT-HAND Y-AXIS TITLE (UPTO 20 CHAR.):'/
1      ' 0      1      2'/
1      ' 12345678901234567890' /)
READ(5,165) (BTITLE(I),I=1,20)
180  CONTINUE
C..
OPEN(UNIT=1,TYPE='OLD',ACCESS='DIRECT',SHARED,READONLY,
&    NAME=PRMFIL,RECL=16)
OPEN(UNIT=2,TYPE='OLD',ACCESS='DIRECT',SHARED,READONLY,
&    NAME=DATFIL,RECL=16)
OPEN(UNIT=3,TYPE='OLD',ACCESS='DIRECT',SHARED,READONLY,
&    NAME=CLCFIL,RECL=16)
OPEN(UNIT=4,TYPE='OLD',ACCESS='DIRECT',SHARED,READONLY,
&    NAME=TCSFIL,RECL=16)
OPEN(UNIT=7,TYPE='OLD',ACCESS='DIRECT',SHARED,READONLY,
&    NAME=GLUFIL,RECL=16)

C..
DO 200 J=1,ILAST
C
READ(2'J) IREC,ETIME,AN2EXT,AO2EXT,CO2EXT,H2OEXT,
&    ETHEXT,AH2EXT,NH3EXT,AN2INL,AO2INL,CO2INL,H2OINL,
&    ETHINL,AH2INL,NH3INL
C
READ(3'J) IREC,CYEAR,CMON,CDAY,CHR,CMIN,CSEC,
&    ETIME,AQVOL,AIRF,OUR,CO2ER,TOTO2,TOTCO2,
&    RSPQUO
C
READ(4'J) IREC,ETIME,PFERM,DO2CON,PLIQO2,PGASO2,KLA,
&    RFLOW1,CELL,AON,ZGLU,ARATE,ARUN,AUSED,
&    SCNINT,AIRFLG
C
READ(7'J) AINOC,ACELL,SLOPE,AINTER,AGLUC,APUMP,DIFF,
&    SGLU,VOLCO2,FGLU
C
C*****

```

```

C
  X(J)=VALUE(IX)
  YA(J)=VALUE(IYA)
  TLAST=ETIME
  YB(J)=VALUE(IYB)
C
200  CONTINUE
     CLOSE(UNIT=1)
     CLOSE(UNIT=2)
     CLOSE(UNIT=3)
     CLOSE(UNIT=4)
     CLOSE(UNIT=7)
C
C CHECK TO SEE IF DATA POINTS ARE TO BE DISCRETE
C
  WRITE(5,210)
210  FORMAT(/,' DISCRETE DATA POINTS? [YES=1, NO=0]: ', $)
     READ(5,*)ICHOOS
     IF(ICHOOS.EQ.1) THEN
       CALL PTDATA(ILAST,X,YA,YB,XMIN,XMAX,YAMIN,YAMAX,
&         YBMIN,YBMAX)
       GOTO 215
     ENDIF
     CALL FPLT3 (ILAST,X,YA,YB,XMIN,XMAX,YAMIN,YAMAX,YBMIN,YBMAX)
215  CONTINUE
C.. MAKE A HARD COPY OF THE PLOT
C   WRITE(5,220)
C220  FORMAT(/' MAKE A HARD COPY OF THE PLOT? YES=1, NO=0: ', $)
C     READ(5,*) ISCR
C     IF(ISCR.NE.1) GO TO 250
C     CALL CPYSCR
C250  CONTINUE
C..
  STOP
  END

```

SUBROUTINE CHG080(IUNIT)

C**** SUBROUTINE TO SET LA50 OR DEC IV HORIZ PITCH TO 80 CHAR

BYTE IOST(4)
BYTE ASK(8),MSG(11),RPY(1)
INTEGER IOBASK(6)

DATA IOBASK(2)/4/

CALL GETADR (IOBASK(1),ASK(1))

ASK(1)="033
ASK(2)="133
ASK(3)="061
ASK(4)="167

CALL WTQIO("410,IUNIT,1,1,IOST,IOBASK,IDS)
WRITE(5,2) IOST,IDS,IOBASK,ASK
2 FORMAT(' ASK:',4I3,2X,I2,2X,I6,5I2/' ',8O4)

RETURN
END

SUBROUTINE CHG132(IUNIT)

C**** SET LA50 OR DEC IV OR VT100 HORIZ PITCH TO 132 CHAR

BYTE IOST(4)

BYTE ASK(8)

INTEGER IOBASK(6)

DATA IOBASK(2)/4/

CALL GETADR (IOBASK(1),ASK(1))

ASK(1)="033

ASK(2)="133

ASK(3)="064

ASK(4)="167

CALL WTQIO("410,IUNIT,1,1,IOST,IOBASK,IDS)

WRITE(5,2) IOST,IDS,IOBASK,ASK

2 FORMAT(' ASK:',4I3,2X,I2,2X,I6,5I2/' ',8O4)

RETURN

END

SUBROUTINE VOLOUT(CN, IAO, VOLT)

C Converts VOLT to the TCS hexadecimal ascii format and
 C puts both the decimal and hexadecimal ascii values into
 C the TCOMON location for channel 8 of block 3 in TCS unit 1
 C Analog Output board 1

c

c AUT: greg O'Connor

c DAT: JULY 2, 1984

c VAR: CN = channel number 1 to 8

c IAO = analog output number 1 or 2

c VOLT = analog output voltage

REAL VOLT

BYTE CN, IAO

INCLUDE 'DL0:[5,5]TCS000.COM'

INCLUDE 'DL0:[5,5]TCS001.COM'

INCLUDE 'DL0:[5,5]TCS002.COM'

INCLUDE 'DL0:[5,5]TCS003.COM'

INCLUDE 'DL0:[5,5]TCSAO1.COM'

INCLUDE 'DL0:[5,5]TCSAO4.COM'

C decimal value of output parameter = VOLT

DOPAO(CN,IAO) = VOLT

C TCS subroutine converts decimal to hexadecimal ascii

CALL CONVDAH(DOPAO(CN,IAO) ,NDPAO(CN,IAO),OPAO(1,CN,IAO))

RETURN

END

```

      SUBROUTINE FPLT3 (N,X,YL,YR,XMIN,XMAX,YLMIN,YLMAX,YRMIN,YRMAX)
C
C SUBROUTINE TO PLOT DATA.
C
      BYTE XTITLE(21),YLTITL(21),YRTITL(21)
      BYTE BATCHN(6)
      INTEGER IYEAR,IMON,IDAY,IHR,IMIN,ISEC
      BYTE ATEXT1(7),ATEXT2(30)
      COMMON/BTHDTA/ BATCHN,IYEAR,IMON,IDAY,ILBL,ISHADE
      COMMON/TITLES/ XTITLE,YLTITL,YRTITL
      DIMENSION X(1000),YL(1000),YR(1000)
C..
      IF(ILBL.EQ.2) GO TO 9
C..
      ENCODE (7,7,ATEXT1) BATCHN
7      FORMAT(6A1)
      ATEXT1(7)=0
C      WRITE(5,20) ATEXT1
      GO TO 50
C..
9      ENCODE (30,10,ATEXT2) BATCHN,IMON,IDAY,IYEAR
10     FORMAT(6A1,14X,I2,'/',I2,'/',I2,2X)
      ATEXT2(30)=0
C      WRITE(5,20) ATEXT2
C20    FORMAT(' ',30A1)
C..
50     CONTINUE
      CALL INITGR (5)
      CALL CLRTXT
      CALL CLRSCR
C..
      CALL DPAPER ('LIN',5,1,'LIN',5,1,'GRAY3')
C..
C..
      CALL LNAXIS ('XB',XTITLE,XMIN,XMAX,.TRUE.)
C..
      CALL LNAXIS ('YL',YLTITL,YLMIN,YLMAX,.TRUE.)
C..
      CALL LNAXIS ('YR',YRTITL,YRMIN,YRMAX,.TRUE.)
C..
      IF(ISHADE.EQ.1) GO TO 800
C..
      CALL PDATA (N,X,YL,'L','GRAY2',,1,,,)
      CALL PDATA (N,X,YR,'R','GRAY3',,1,,,)
      GO TO 890
C..
800    CONTINUE
      CALL PDATA (N,X,YL,'L','GRAY3',,1,,,)
      CALL PDATA (N,X,YR,'R','GRAY2',,1,,,)
      CALL PDATA (N,X,YR,'R','GRAY2',,6,,.TRUE.,)
C..

```



```
890  IF(ILBL.EQ.2) GO TO 900
C..
    CALL LINETX(5,67,ATEXT1)
    GO TO 949
C..
900  CONTINUE
    CALL SWINDO (0.,0.,1000.,625.)
    CALL MOVE (0.,625.)
    CALL STXSIZ (3,4)
    CALL TEXT (ATEXT2)
    GO TO 949
C..
949  WRITE(5,950)
950  FORMAT('/ COPY TO LA50? Y/N ', $)
    READ(5,951) IANS
951  FORMAT(A1)
    IF(IANS.EQ.'N') RETURN
C..
    CALL CPYSCR
C..
C.. PAUSE TO ALLOW PLOT TO BE COPIED.
C..
990  READ(5,1000) IDUM
1000 FORMAT(A1)
C..
    RETURN
    END
```

```

      SUBROUTINE PTDATA(N,X,YL,YR,XMIN,XMAX,YLMIN,YLMAX,YRMIN,YRMAX)
C
C MODIFIED TO JUST PLOT DATA POINTS 34/1/86 BY JIM MCMILLAN
C
C SUBROUTINE TO PLOT DATA.
C
      BYTE XTITLE(21),YLTITL(21),YRTITL(21)
      BYTE BATCHN(6)
      INTEGER IYEAR,IMON,IDAY,IHR,IMIN,ISEC
      BYTE ATEXT1(7),ATEXT2(30)
      COMMON/BTHDTA/ BATCHN,IYEAR,IMON,IDAY,ILBL,ISHADE
      COMMON/TITLES/ XTITLE,YLTITL,YRTITL
      DIMENSION X(1000),YL(1000),YR(1000)
C..
      IF(ILBL.EQ.2) GO TO 9
C..
      ENCODE (7,7,ATEXT1) BATCHN
7     FORMAT(6A1)
      ATEXT1(7)=0
C     WRITE(5,20) ATEXT1
      GO TO 50
C..
9     ENCODE (30,10,ATEXT2) BATCHN,IMON,IDAY,IYEAR
10    FORMAT(6A1,14X,I2,'/',I2,'/',I2,2X)
      ATEXT2(30)=0
C     WRITE(5,20) ATEXT2
C20   FORMAT(' ',30A1)
C..
50    CONTINUE
      CALL INITGR (5)
      CALL CLRTXT
      CALL CLRSCR
C..
      CALL DPAPER ('LIN',5,1,'LIN',5,1,'GRAY3')
C..
C..
      CALL LNAXIS ('XB',XTITLE,XMIN,XMAX,.TRUE.)
C..
      CALL LNAXIS ('YL',YLTITL,YLMIN,YLMAX,.TRUE.)
C..
      CALL LNAXIS ('YR',YRTITL,YRMIN,YRMAX,.TRUE.)
C..
      IF(ISHADE.EQ.1) GO TO 800
C..
      CALL PDATA (N,X,YL,'L','GRAY2',1,0,,,)
      CALL PDATA (N,X,YR,'R','GRAY3',2,0,,,)
      GO TO 890
C..
800   CONTINUE
      CALL PDATA (N,X,YL,'L','GRAY3',1,0,,,)
      CALL PDATA (N,X,YR,'R','GRAY2',2,0,,,)

```

```
      CALL PDATA (N,X,YR,'R','GRAY2',,6,,TRUE.,)
C..
890   IF(ILBL.EQ.2) GO TO 900
C..
      CALL LINETX(5,67,ATEXT1)
      GO TO 949
C..
900   CONTINUE
      CALL SWINDO (0.,0.,1000.,625.)
      CALL MOVE (0.,625.)
      CALL STXSIZ (3,4)
      CALL TEXT (ATEXT2)
      GO TO 949
C..
949   WRITE(5,950)
950   FORMAT('/' COPY TO LA50? Y/N ',')
      READ(5,951) IANS
951   FORMAT(A1)
      IF(IANS.EQ.'N') RETURN
C..
      CALL CPYSCR
C..
C.. PAUSE TO ALLOW PLOT TO BE COPIED.
C..
990   READ(5,1000) IDUM
1000  FORMAT(A1)
C..
      RETURN
      END
```

APPENDIX B

Paragon Data Acquisition and Control Program

The Paragon program consists of a main database, which contains all the graphically connected programming blocks, several C subroutines, which interact with the main database, and a series of graphical templates, which display the data to the screen.

The display templates developed were as follows:

- 1) The gas composition display of all channels of the mass spectrometer (Developed by Intec Controls Corporation).
- 2) Contains all the initial settings required by the program.
- 3) Summary of mass spectrometer data of selected input and output channels.
- 4) Readings from fermenter, pH, DO, temperature etc.
- 5) Mass transfer data.
- 6) Mass balance on glucose.
- 7) Readings from balances.
- 8) Selection of glucose control strategy.
- 9) Warning/error checking panel.
- 10) Cell concentration and growth rate estimation.
- 11) Inlet gas mixing control strategy.
- 12) Fishmeal feeding strategy.
- 13) Comparison of on-line data with the model.

GO PAUSE

MIT FERMENTATION STUDIES

CHNL	TIME	HOLD	N2	O2	CO2	H2O	ETOH	H2	NH3	CH4
1	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
2	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
3	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
4	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
5	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
6	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
7	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
8	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
9	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
10	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
11	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
12	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
13	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
14	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
15	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
16	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
17	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
18	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00
19	00000	00	000.00	000.00	000.00	000.00	000.00	000.00	000.00	000.00

01:00:00 Mon Jan 01 1988

INITIAL SETTINGS

TIME OF INOCULATION		MASS SPEC CHANNELS		BALANCES: INITIAL WEIGHT	
YEAR	<input type="text" value="00."/>	INLET	<input type="text" value="00."/>	NaOH	<input type="text" value="0000.00"/> g
MONTH	<input type="text" value="00."/>	OUTLET	<input type="text" value="00."/>	GLUCOSE	<input type="text" value="0000.0"/> g
DAY	<input type="text" value="00."/>			FERMENTER EMPTY	<input type="text" value="00.00"/> kg
HOUR	<input type="text" value="00."/>	GLUCOSE CONC IN FEED TANK	<input type="text" value="000.0"/> g/l		
MINUTE	<input type="text" value="00."/>				
SECOND	<input type="text" value="00."/>				
				AMOUNT OF GLUCOSE IN THE FERMENTER PRIOR TO INOCULATION	<input type="text" value="00.0"/> g
CELL CONC IN FERMENT AFTER INOC	<input type="text" value="00.0"/> g/l		ferm vol 0.00	INITIAL LIQUID VOLUME IN FERMENTER	<input type="text" value="0.00"/> l
DISSOLVED O2 AT SATURATION	<input type="text" value="000."/> units	GLUCOSE ADJ FROM HPLC		YIELD COEFFICIENT mmols CO2 EVOLVED PER g GLUCOSE CONSUMED	<input type="text" value="00.00"/>
PRESSURE IN FERMENTER	<input type="text" value="00.0"/> (psia)		<input type="text" value="000.0"/>		

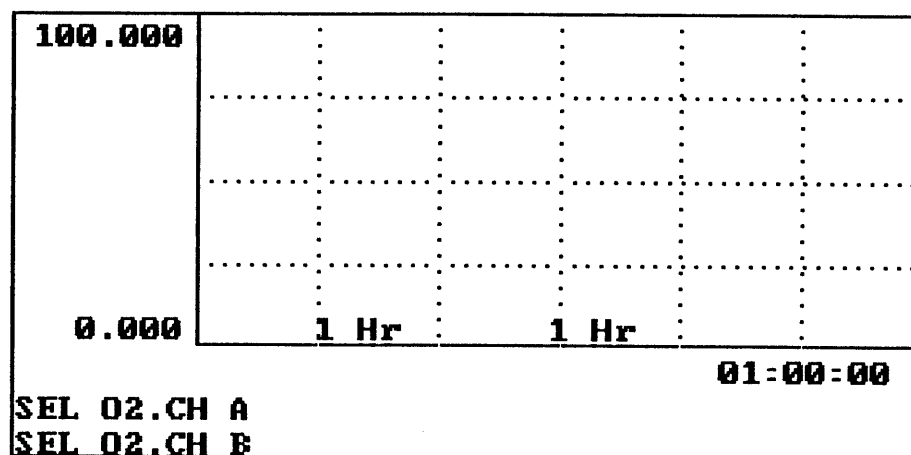
ELAPSED TIME 00.000

NUMBER OF SCANS 0000.
(HISTORY FILES)

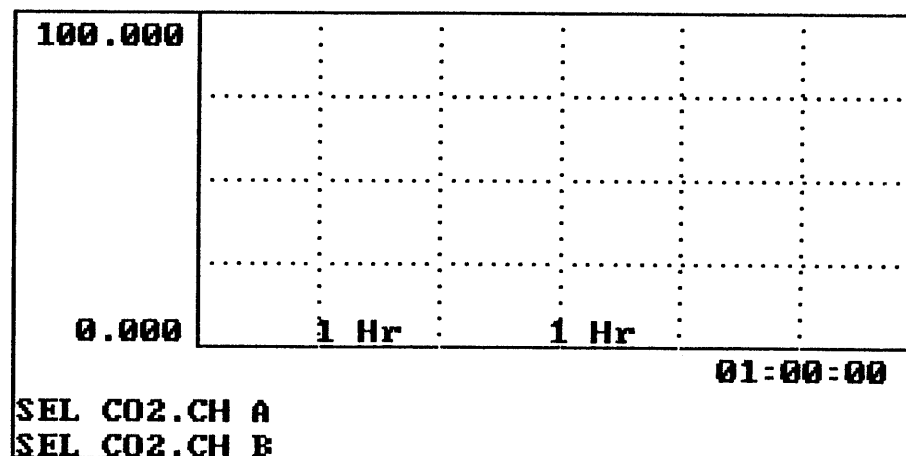
MASS SPEC DATA SUMMARY

GAS TYPE	INLET CHAN 00.	OUTLET CHAN 00.
N2	00.000	00.000
O2	00.000	00.000
CO2	00.000	00.000
H2O	00.000	00.000
ETOH	00.000	00.000
H2	00.000	00.000
NH3	00.000	00.000
CH4	00.000	00.000

OXYGEN CONCENTRATIONS



CO2 CONCENTRATIONS



FERMENTER OPERATION

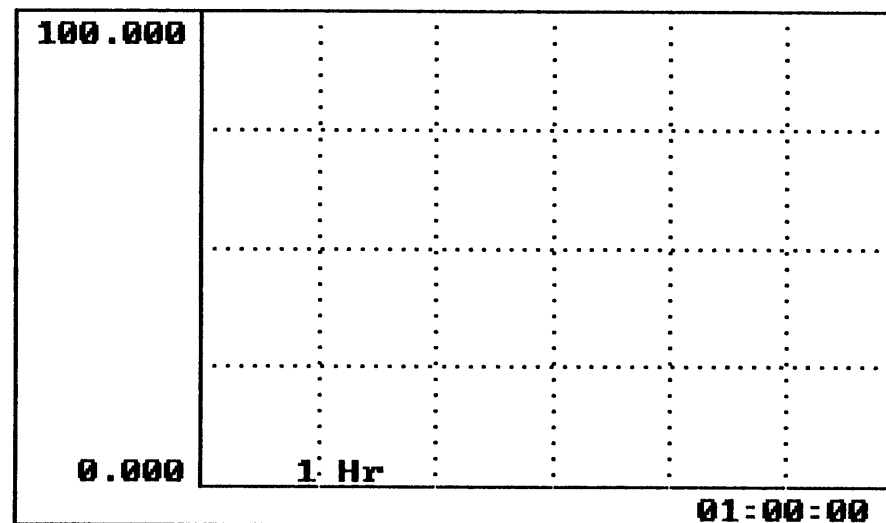
FERMENTER VOL	0.00	l
TEMPERATURE	00.0	C
pH	00.00	units
DO RAW READING	000.0	units
DO SATURATION	000.0	units
DO COMPARED TO SATURATION	000.0	% satn

PC TIME

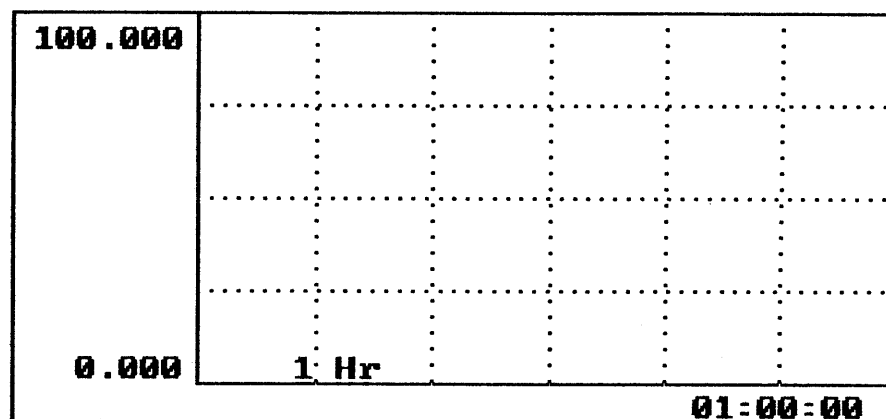
Mon Jan 01 1988

01:00:00

DISSOLVED OXYGEN



PH



OXYGEN & CO2 TRANSFER DATA

OUR 000.00 mmoles O2/1-h

CER 000.00 mmoles CO2/1-h

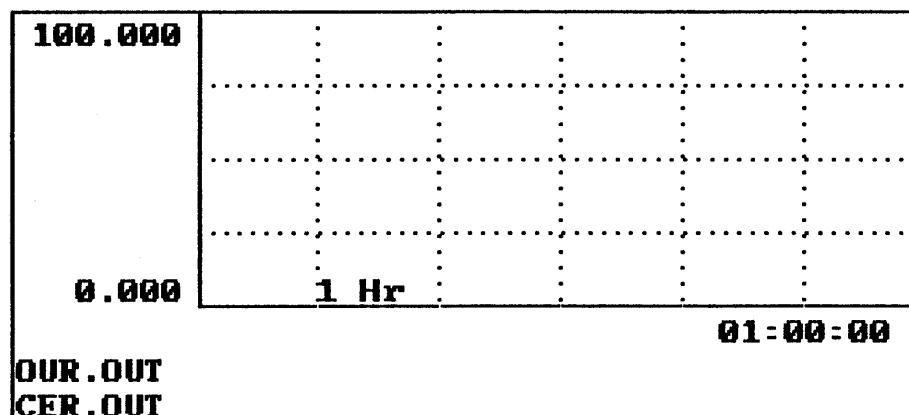
RQ 00.000 mmoles CO2
PER mmole O2

TOTAL CO2 0000.00 mmoles
EVOLVED 0000.00 mmoles/1

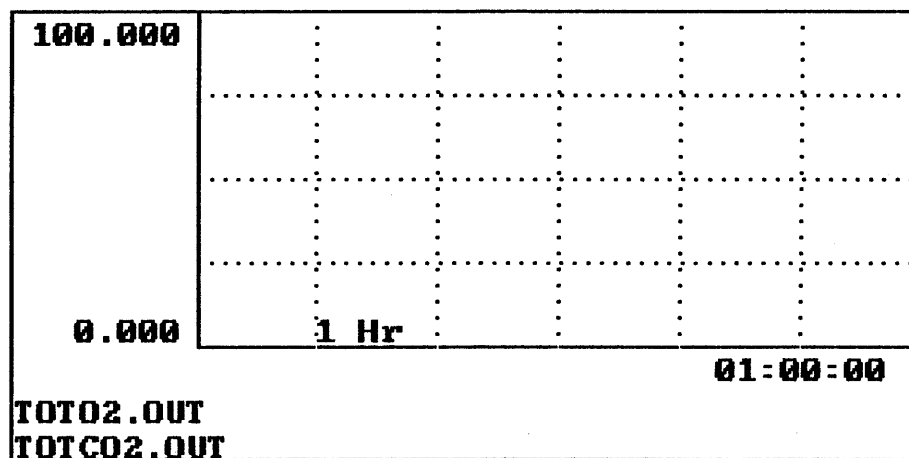
TOTAL O2 0000.00 mmoles
CONSUMED

kla 000.00 mmoles/1-h-atm
000.00 1/h

OUR & CER



TOTAL O2 & TOTAL CO2



GLUCOSE SET POINT

00.0

g/l

GLUCOSE CONTROL STRATEGY

SAMPLE
SCANS
00000.

FERM VOL 1
00.00

TOTAL SAMPLE
VOLUME ml
0000.0

GLUCOSE
INITIAL

g

000.00

GLUCOSE
ADDED

g

000.00

GLUCOSE FEED PUMP



* PUMP ON
* PUMP OFF

ACTUAL
TOTAL ADDED
FM SOLN

00000.0

g

SAMPLE TAKING
INDICATOR
000.

GLUC ADJUST

GLUC COMP (g/l)

000.00

GLUCOSE
SAMPLE

g

000.00

000.00

GLUCOSE
CONSUMED

g

GLUCOSE CONC g/l

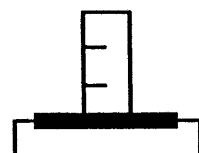
prog	adjust	used
00.00	00.00	00.00

100.000

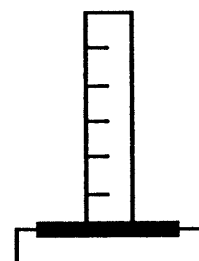
0.000

1 Hr

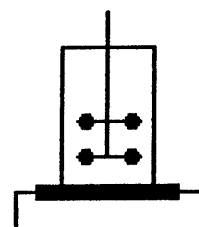
01:00:00



NaOH ADDN
BALANCE
0000.00 g



GLUCOSE ADDN
BALANCE
0000.0 g



FERMENTER
BALANCE
00.00 kg

BALANCES

NaOH ADDED	0000.00	g
GLUCOSE ADDED	0000.0	g
FERMENTER VOL	00.00	kg
FERMENTER EMPTY	00.00	kg

100.000						
0.000	1 Hr					
					01:00:00	
FERM VOL.OUT						
GLUC ADD.OUT						
NaOH ADD.OUT						

SELECTION OF GLUCOSE CONTROL STRATEGY

GLUCOSE KEPT
CONSTANT

GLUCOSE CONTROLLED TO BE THE LEAST TOXIC BUT AT
THE SAME TIME NOT LIMITING GROWTH.

SELECTION: MU FROM ILS: 1 MU FROM CO2: 0 set pt used 00.0

GLUCOSE
SETPOINT

00.0

(g/l)

MU FROM LIGHT SCATTER

GROWTH RATE 0.000 (1/h)
GLUC OURFEED 00.0 (g/l)
GLUC LIMITED 00.0 (g/l)
GLUC SETPT 00.0 (g/l)

MU FROM CO2 DATA

GROWTH RATE 0.000 (1/h)
GLUC OURFEED 00.0 (g/l)
GLUC LIMITED 00.0 (g/l)
GLUC SETPT 00.0 (g/l)

SELECTION: CONSTANT G: 1
NON-TOXIC G: 0

PROG CALC 00.0
ADJUSTMENT 00.0
USED VALUE 00.0

WARNING PANEL

● PROBLEM

● ALL OK

pH < 6.9 ●

pH > 7.1 ●

DO < DOSET ●

DO > 90% ●

TEMP > 35.0 C ●

TEMP < 33.0 C ●

GLUCOSE < 2 g/l ●

GLUCOSE > 12 g/l ●

FLOW TO FERM < 0.5 l/m ●

VOL FERM < 0.5 l ●

VOL FERM > 5.9 l ●

MASS SPEC COMM PROB ●

LIMITATIONS

BASED ON CO2 READING

GLUCOSE LIMITED ●

FISHMEAL LIMITED ●

BASED ON ILS READING

GLUCOSE LIMITED ●

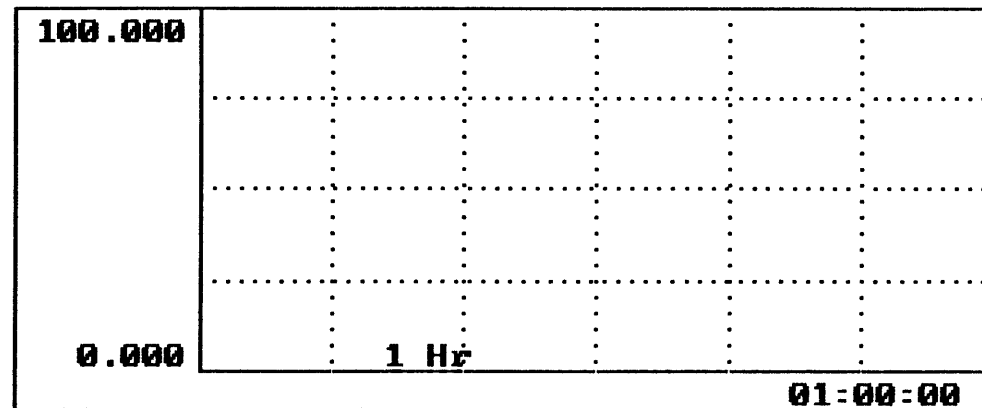
FISHMEAL LIMITED ●

CELL CONC

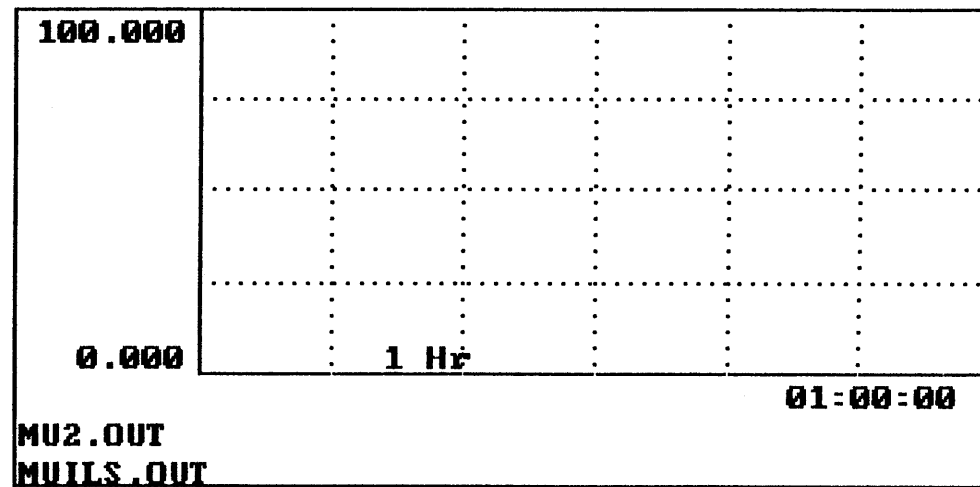
CELLS

INITIAL CONC 00.0 g/l
 CONC NOW 00.0 g/l
 MU (CO2) 0.000 1/h

CELL CONCENTRATION (g/l)



GROWTH RATE CO2 & ILS





ENRICHED OXYGEN FLOW TO THE FERMENTER

GAS MIXING STATUS

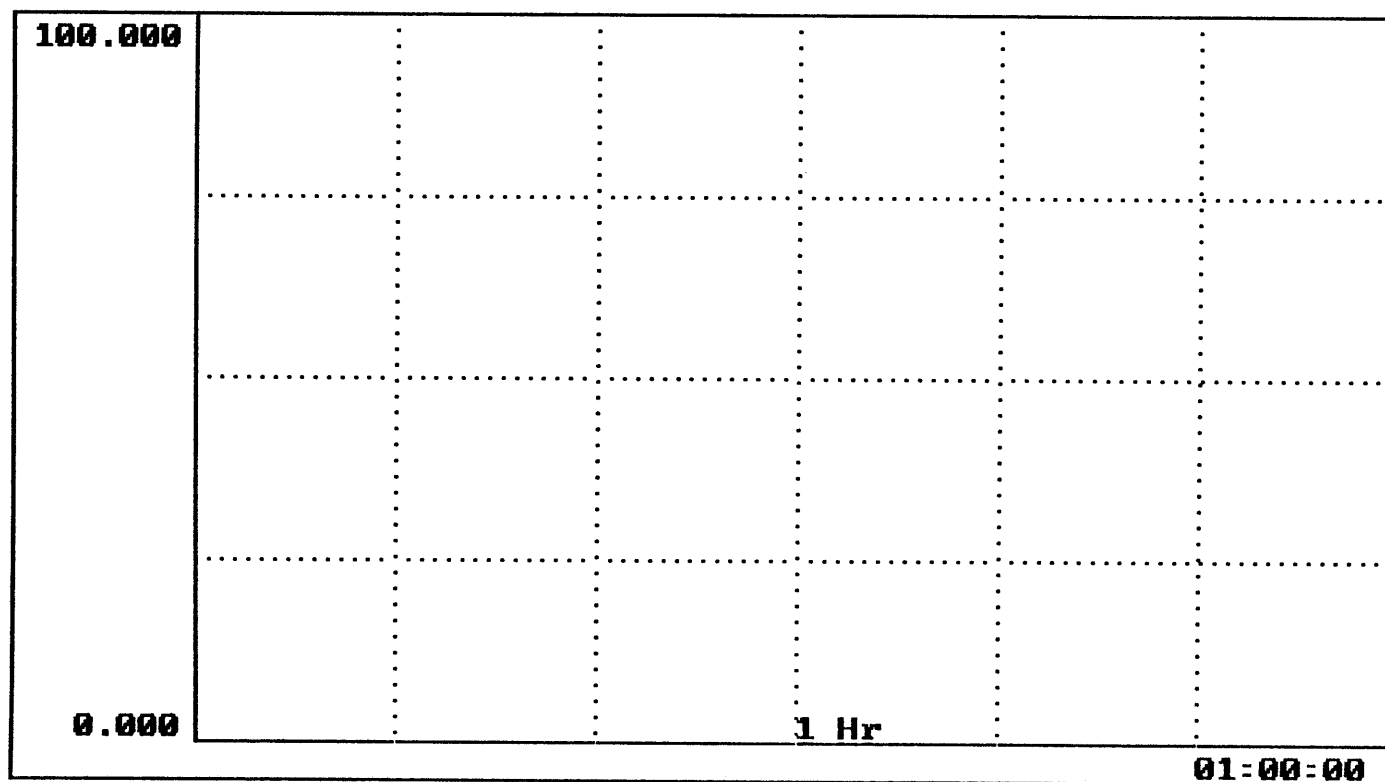
COMPLETELY MANUAL O2 SETPT <input type="text" value="000.000"/> AIR SETPT <input type="text" value="000.000"/> <input type="radio"/>		O2 CANT REACH SET <input type="radio"/>		AIR CANT REACH SET <input type="radio"/>	
REFERENCE AIR FLOW <input type="text" value="000.000"/>		CONTROL AIR ONLY UUM SET PT <input type="text" value="0.000"/> ACTUAL 0.000 FERM VOL 00.00		DO CONTROL <input type="radio"/> DO < SETPT <div> <div></div> <div></div> </div> DO SETPT <input type="text" value="00.00"/> UUM SETPT <input type="text" value="0.000"/> FLOMAX <input type="text" value="00.00"/> GAIN <input type="text" value="00.0000"/> DO % SAT 000.00 DO RAW 000.00	
MANUAL 0 <input type="text" value="N"/> CONTROL 1		<input type="radio"/> OXYGEN CONC IN 00.000 OUT 00.000		<input type="radio"/>	
TOTAL FLO <input type="text" value="000.00"/>		FLO TO FERM <input type="text" value="000.00"/>		AIR CONTROL 0 <input type="text" value="N"/> AIR & O2 CONTROL 1	

OXYGEN				AIR			
RAW SET	000.00	CAL SET	000.00	RAW SET	000.00	CAL SET	000.00
RAW FLO	000.00	CAL FLO	000.00	RAW FLO	000.00	CAL FLO	000.00

 LAG  GROWTH CO2 SETPT <div>000.000</div> % CO2 VALUE 000.000 % BEGINNING GROWTH PHASE 000.000 h LENGTH GROWTH PHASE 000.000 h	FM FEED STRATEGY FEEDFORWARD CONTROL MODEL ESTIMATED DISS FM 00.00 g/l FISHMEAL FEED NOW 000.000 g/l 000.000 g TOTAL 000.000 g/l 000.000 g TO DATE BASED ON ILS DEL D 000.000 g/l NOW 000.000 g/l 000.000 g TOTAL 000.000 g/l 000.000 g TO DATE WHEN I'M GREEN 00. YOU CAN FEED	SAMPLE ml 00000. <div>000.</div>	CO2 DISS FM 00.00 g/l ILS DISS FM 00.00 g/l MU ILS <div>0.000</div> 1/h MU CO2 0.000 1/h P VALUE <div>000.000</div> I VALUE <div>000.000</div> D VALUE <div>000.000</div> BASED ON CO2 DEL D 000.000 g/l NOW 000.000 g/l 000.000 g TOTAL 000.000 g/l 000.000 g TO DATE FERM VOL 00.00 l ONLY ADD FISHMEAL IF I AM BETWEEN 1-7 00.
CO2 DET 0 MANUAL 1 <div>N</div> OVERRIDE BEG GROWTH <div>000.000</div>	CO2 ACT 0 CO2 SET 1 <div>N</div> CO2 VALUE SET <div>000.000</div>	AMOUNT OF FISHMEAL SOLUTION ADDED IN g <div>00000.0</div>	FISHMEAL ACTUALLY ADDED NOW 000.000 g/l <div>000.000</div> g TOTAL 000.000 g/l 000.000 g TO DATE time 01:00:00 Mon Jan 01 1988 elapsed time 000.000 h

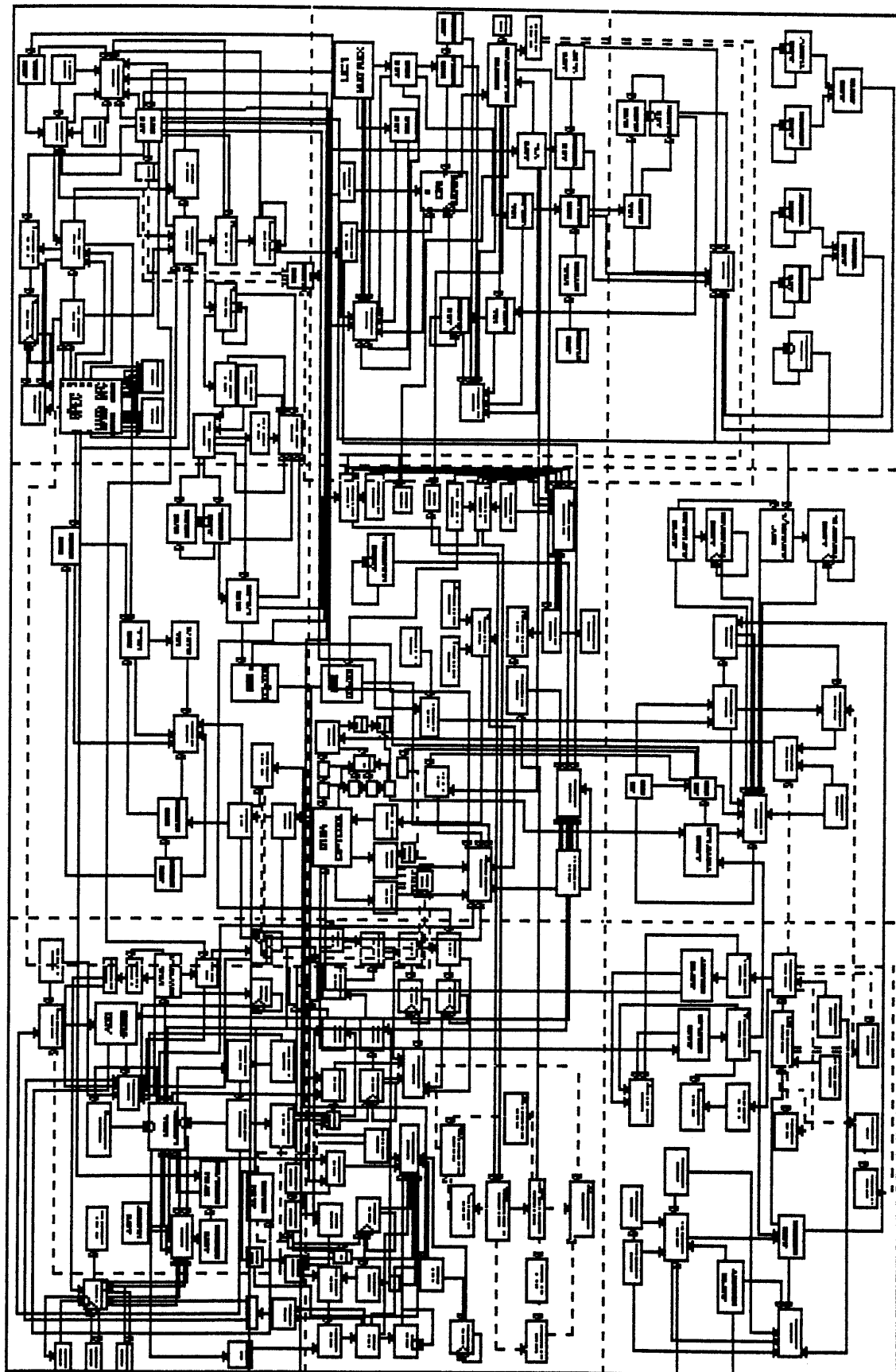
D(CO2)
D(ILS)
D(MODEL)

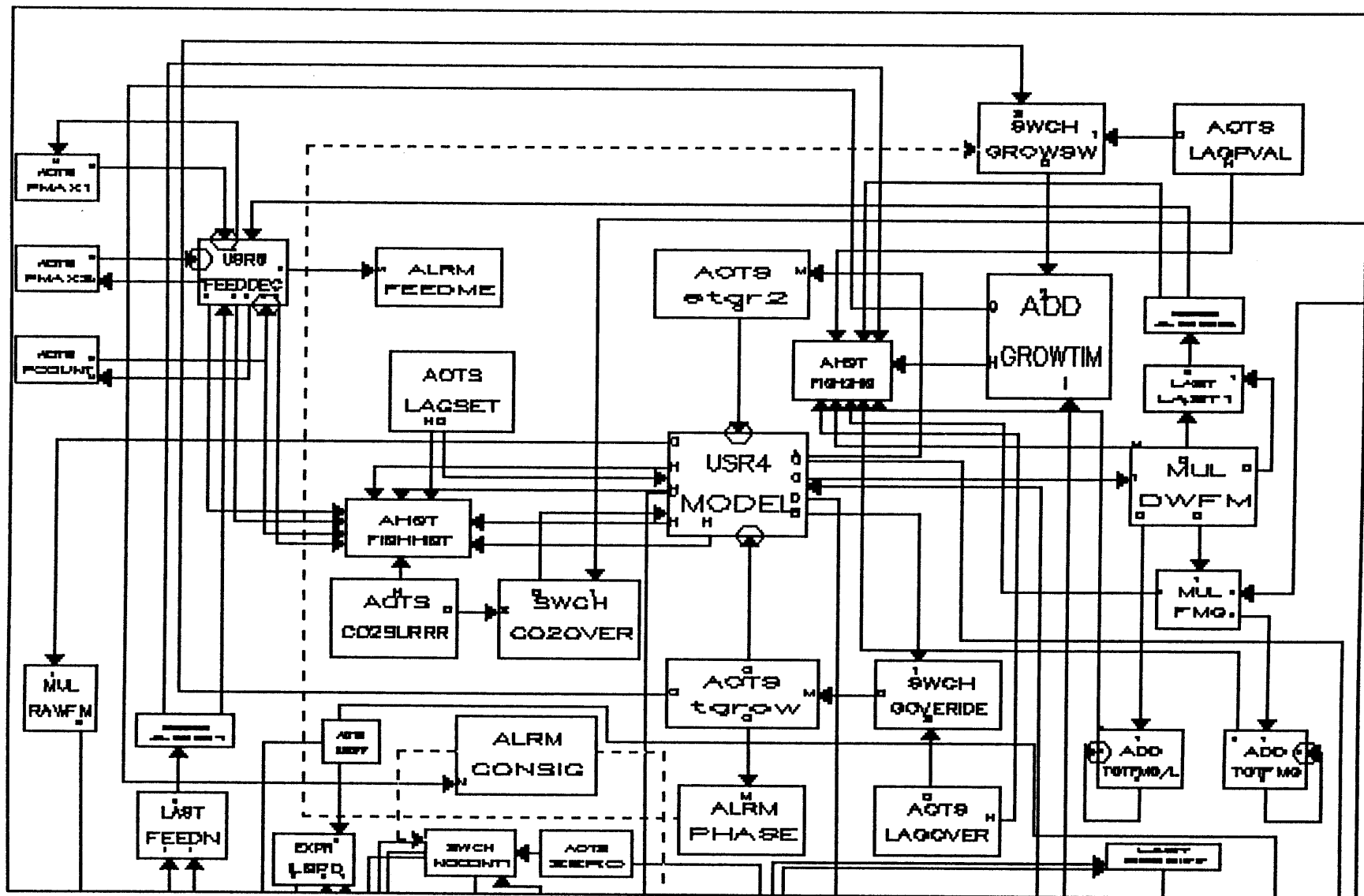
DISSOLVED FISHMEAL CONCENTRATION ---

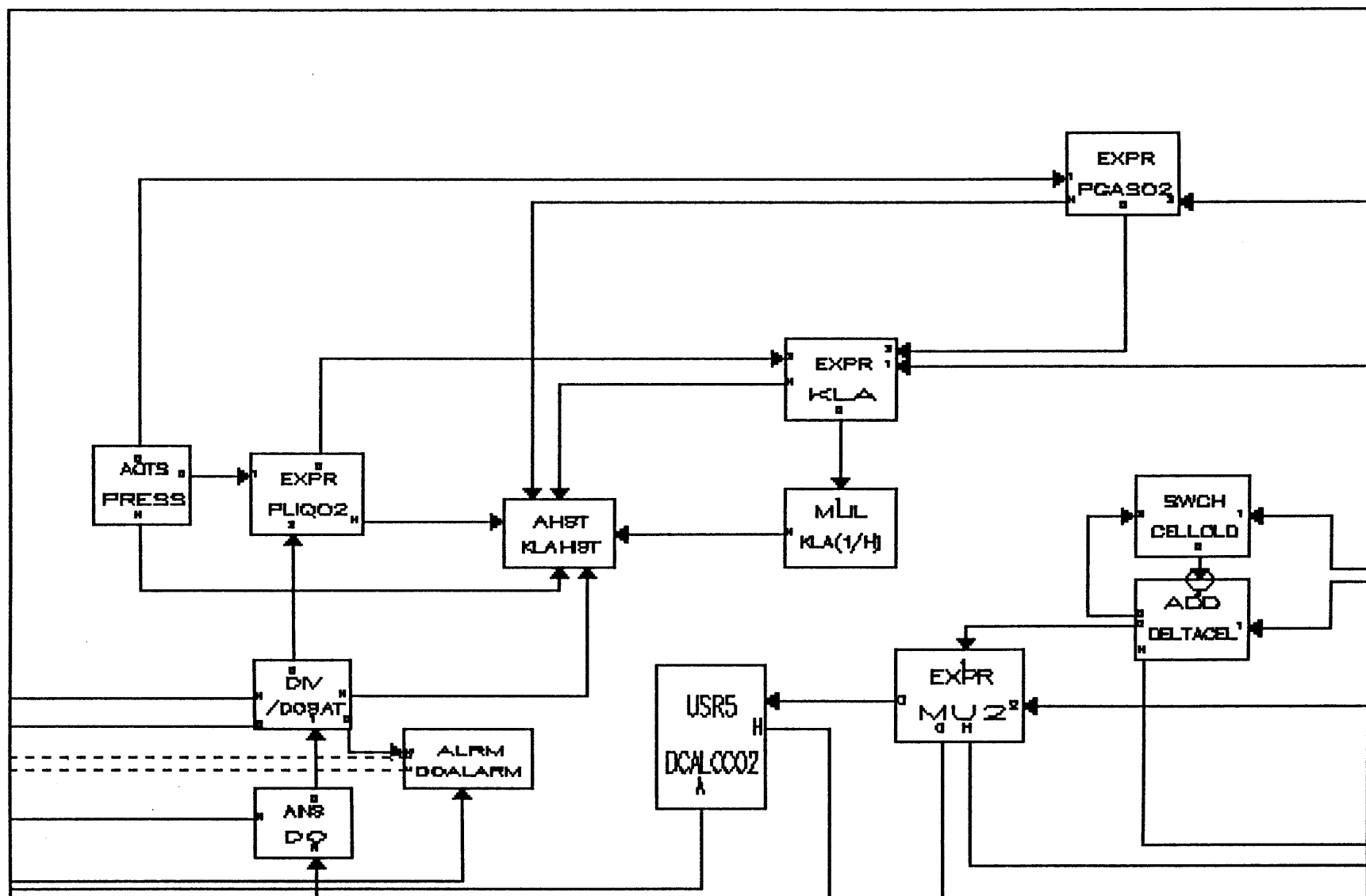


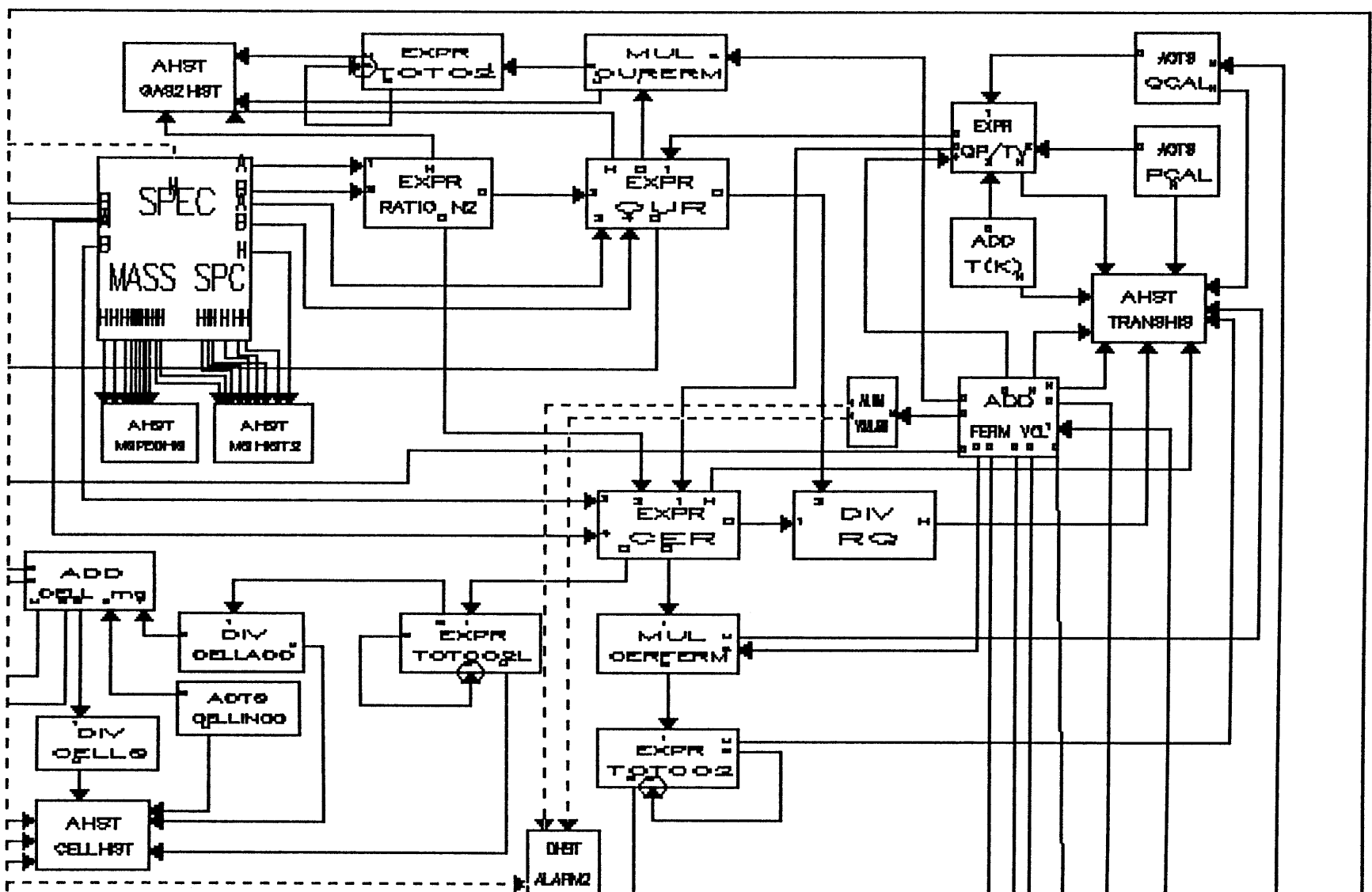
Next is shown the Paragon control database. This is a series of interconnected blocks. This output was printed on a Panasonic laserjet, which was configured to emulate an IBM proprinter. The displays are as follows:

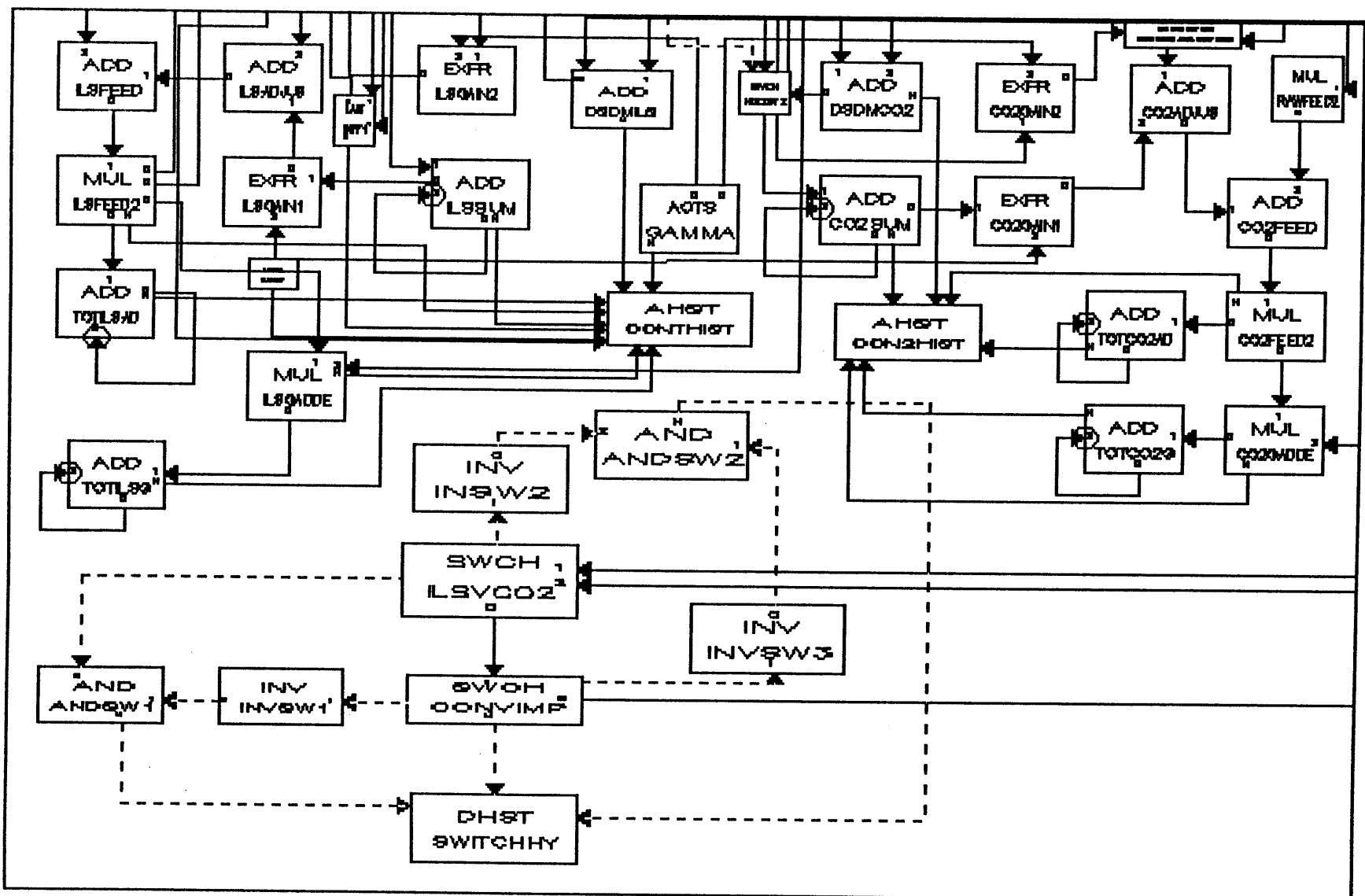
- 1) An overview of the entire network containing nine screens.
- 2) Screens 1-9 enlarged.
- 3) Further enlargements of particular sections of the database
 - glucose feeding strategy
 - the model
 - the PID controller for fishmeal addition
 - the OPTO 22 signal conditioning unit
- 4) Detail inside the MASS SPC block, used to select the data from a particular gas stream from the mass spectrometer.
- 5) Detail inside the FEEDN block, used to calculate the difference between the current value of a variable and its value at the last scan.

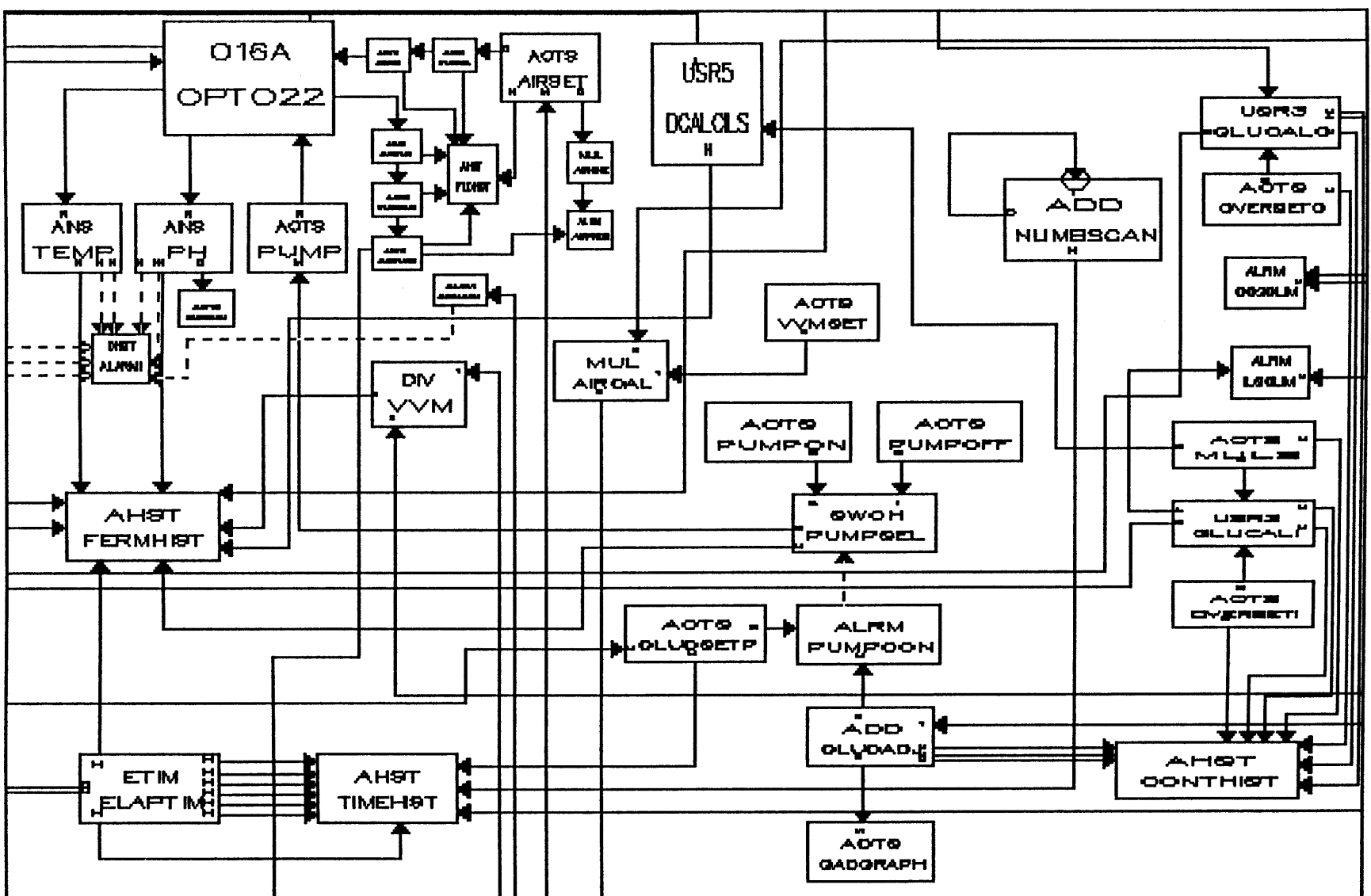


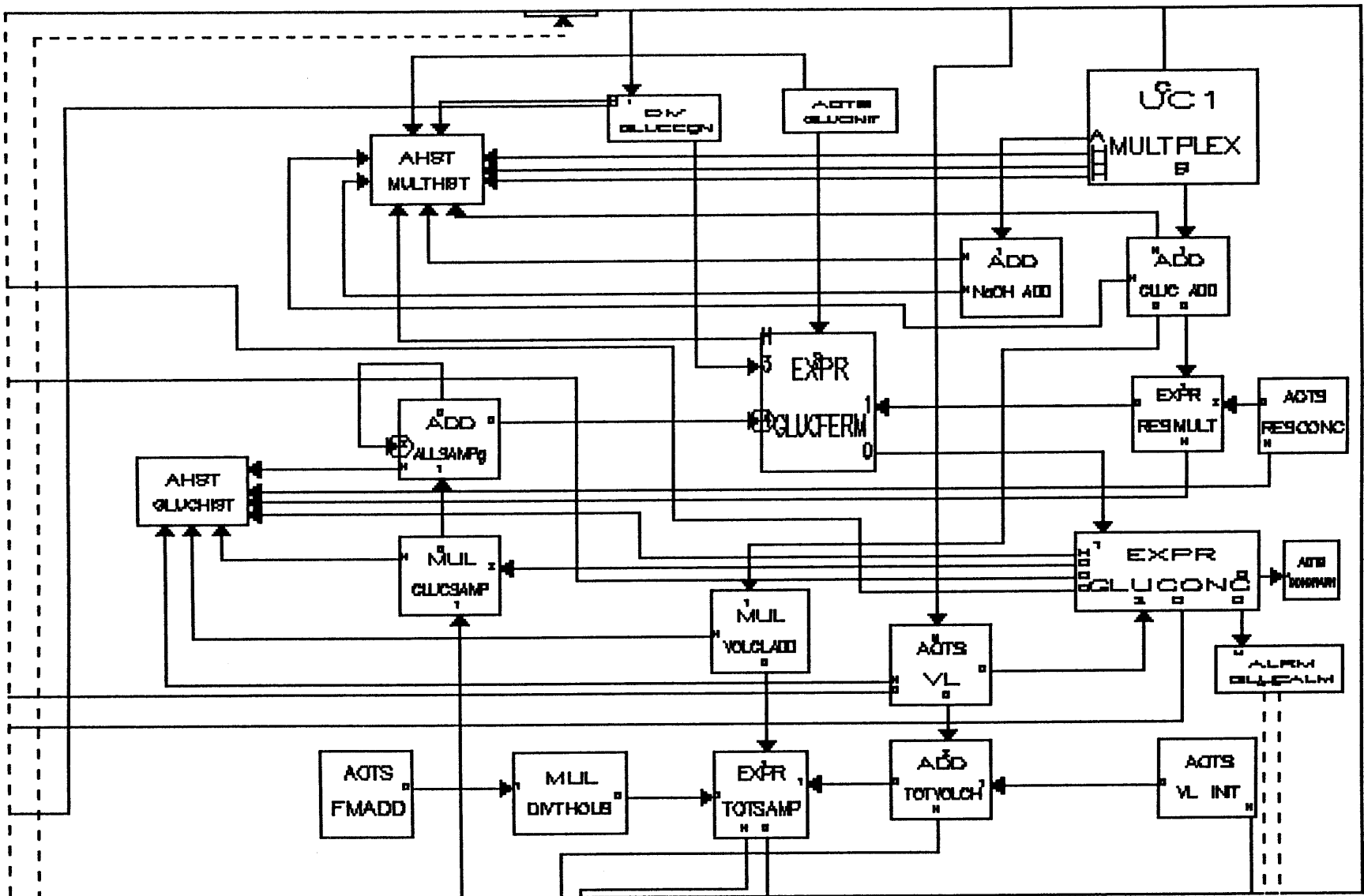


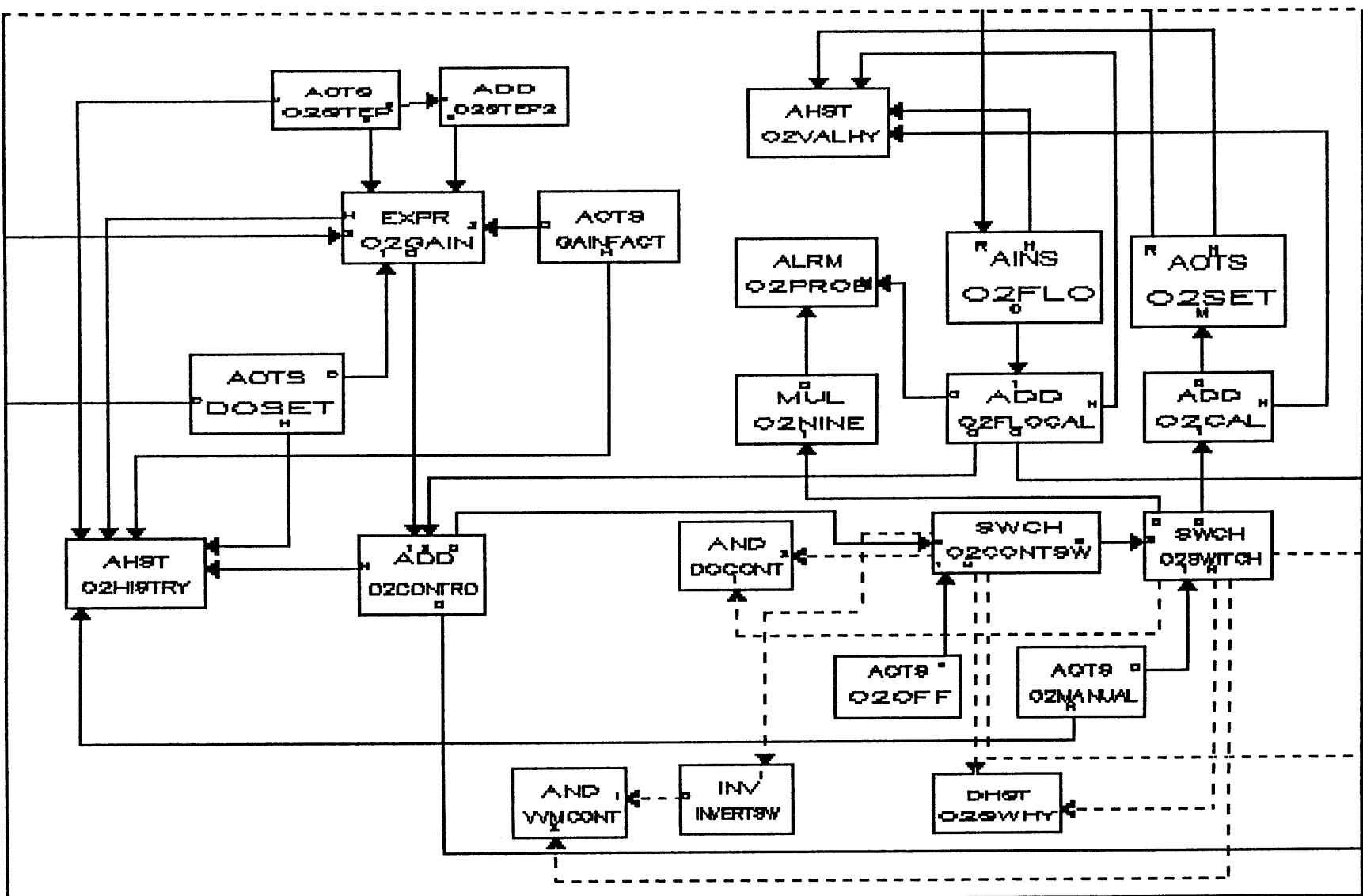


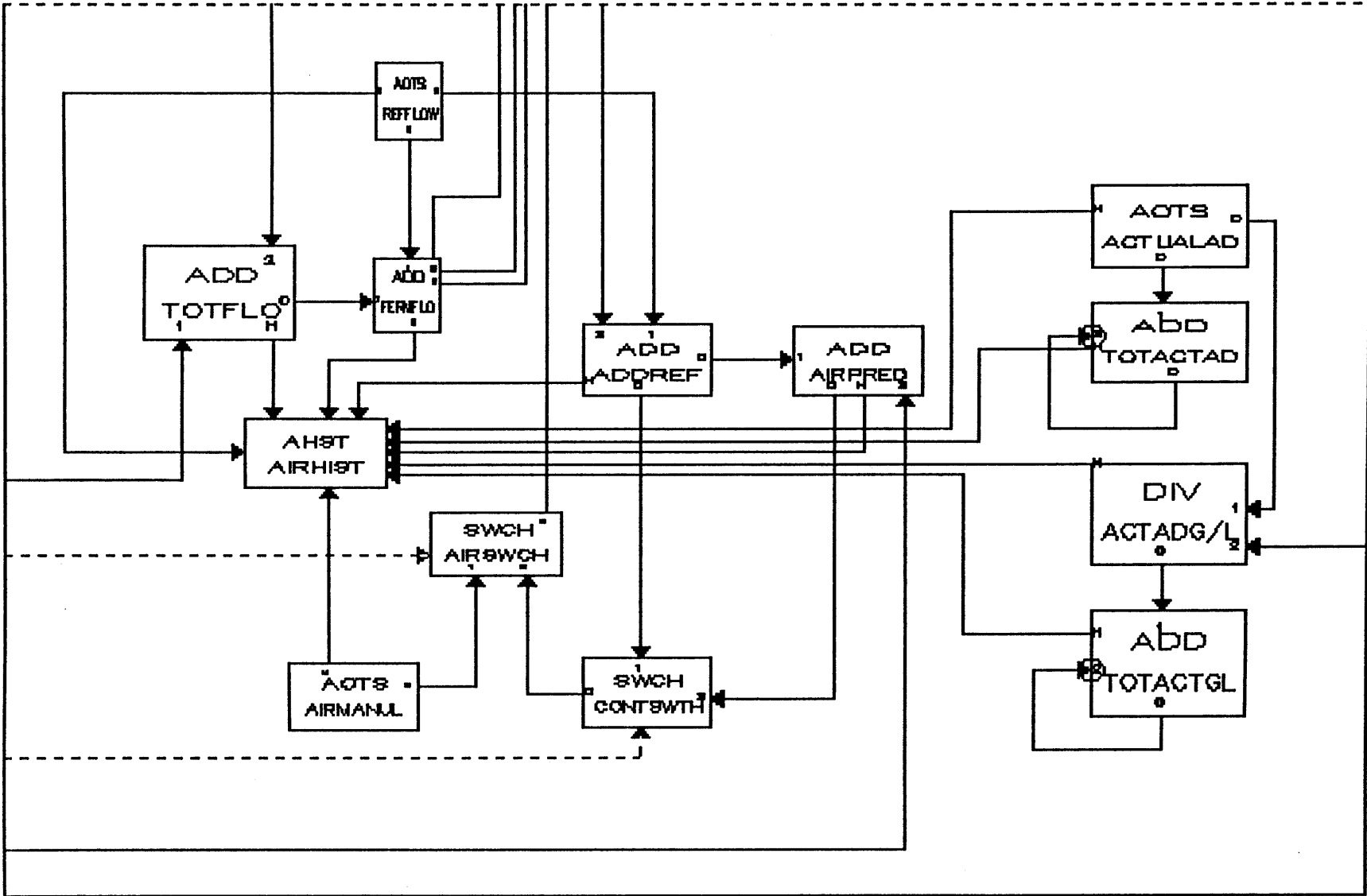


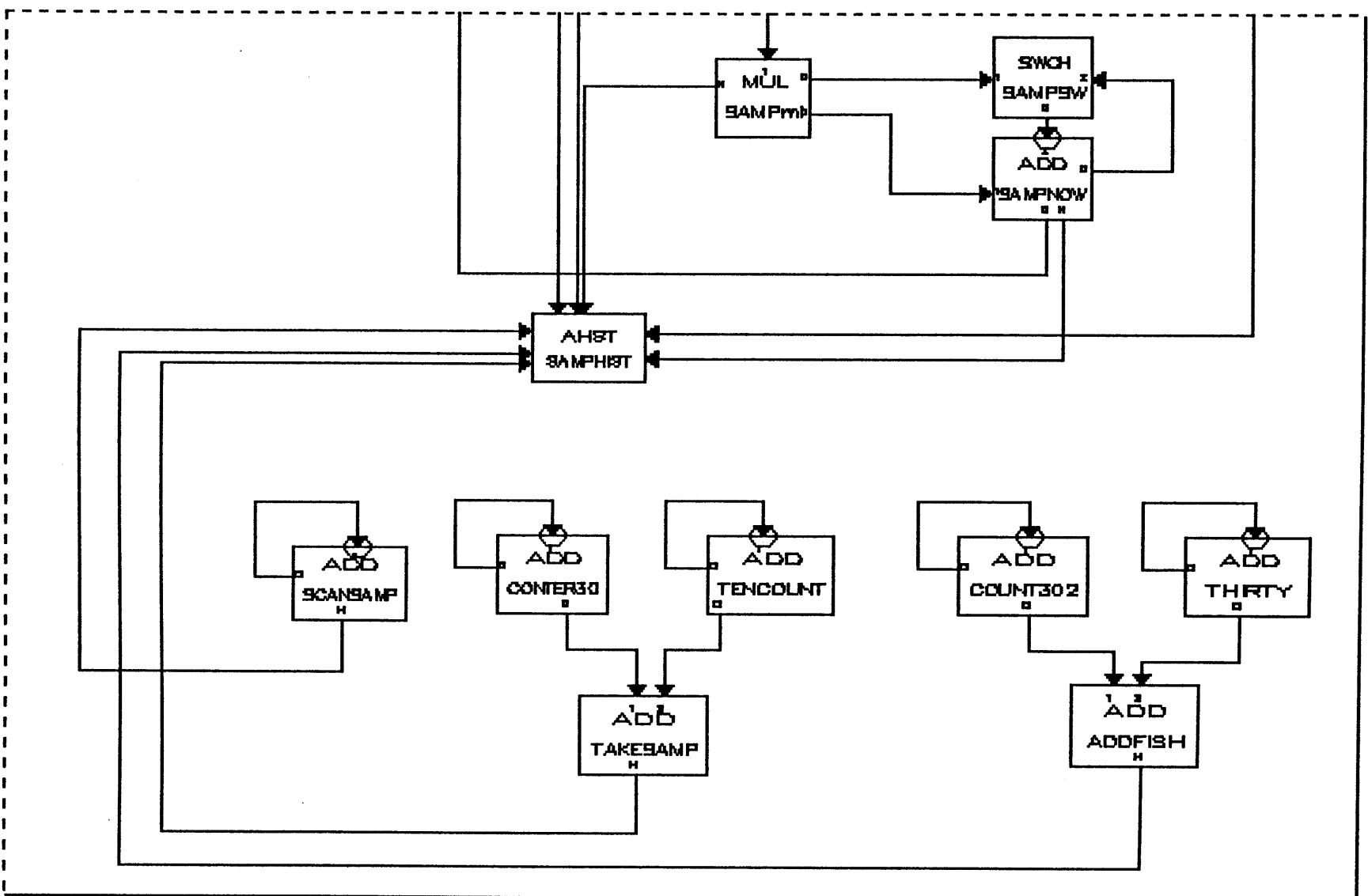


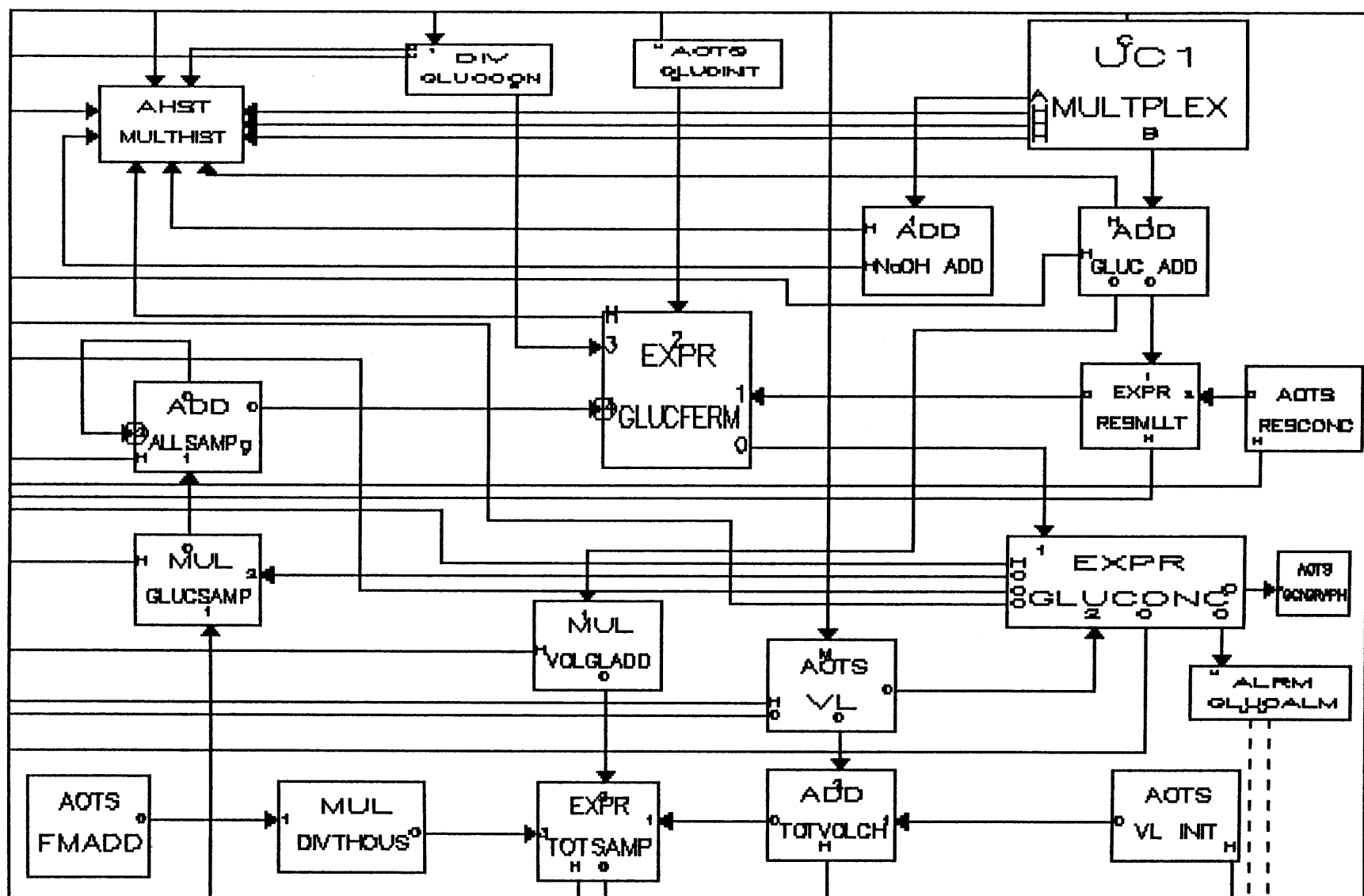


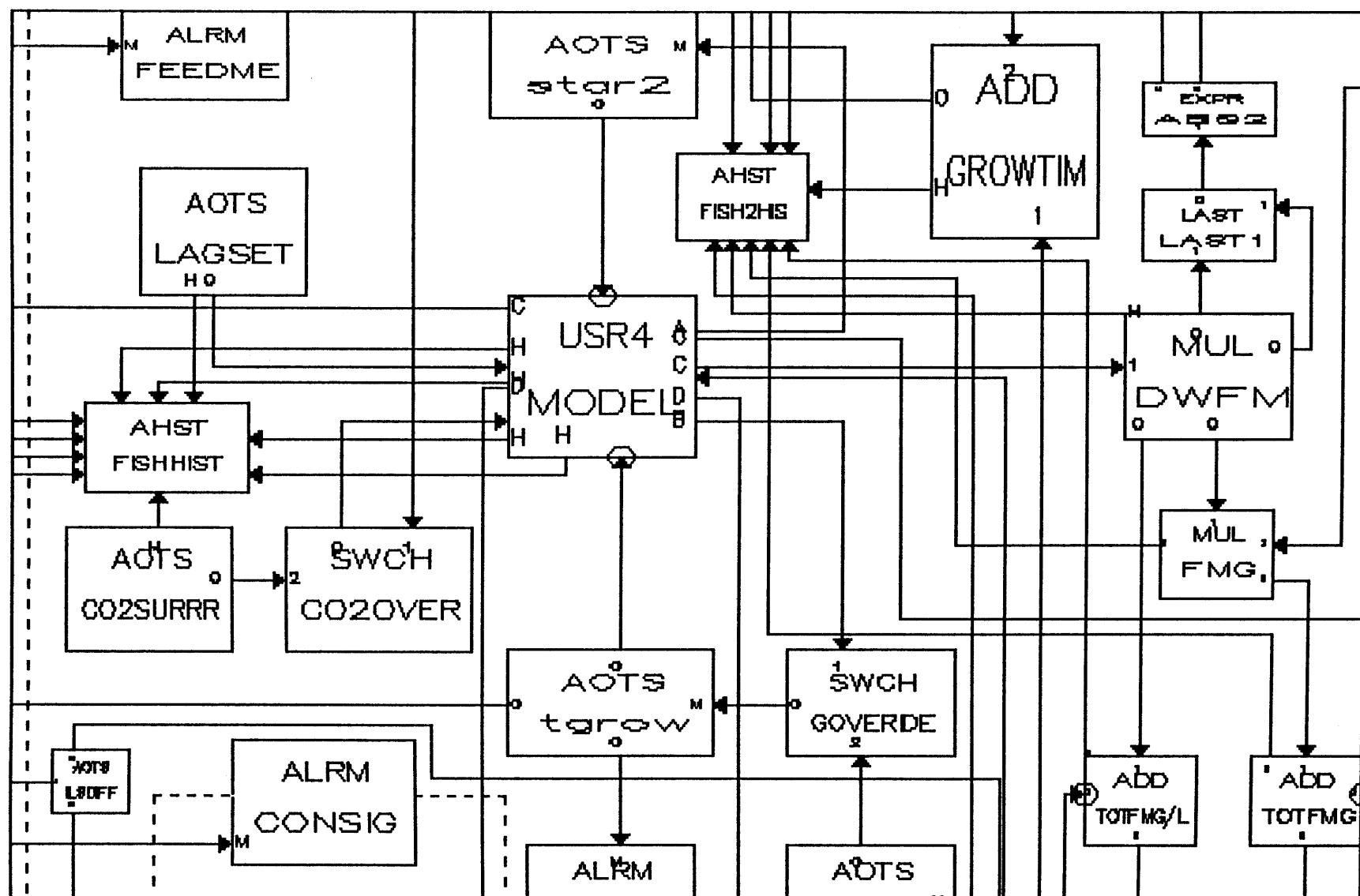


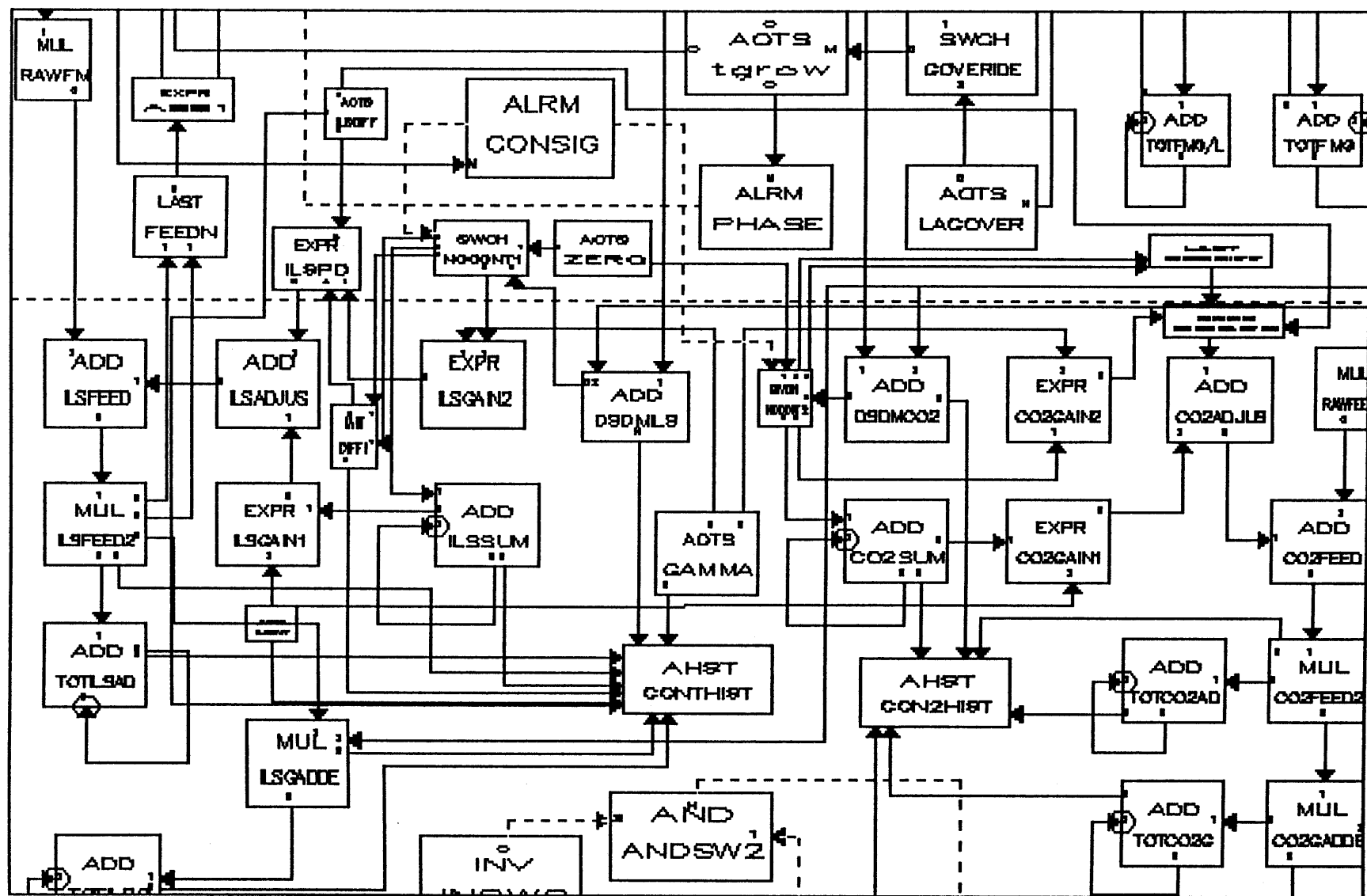


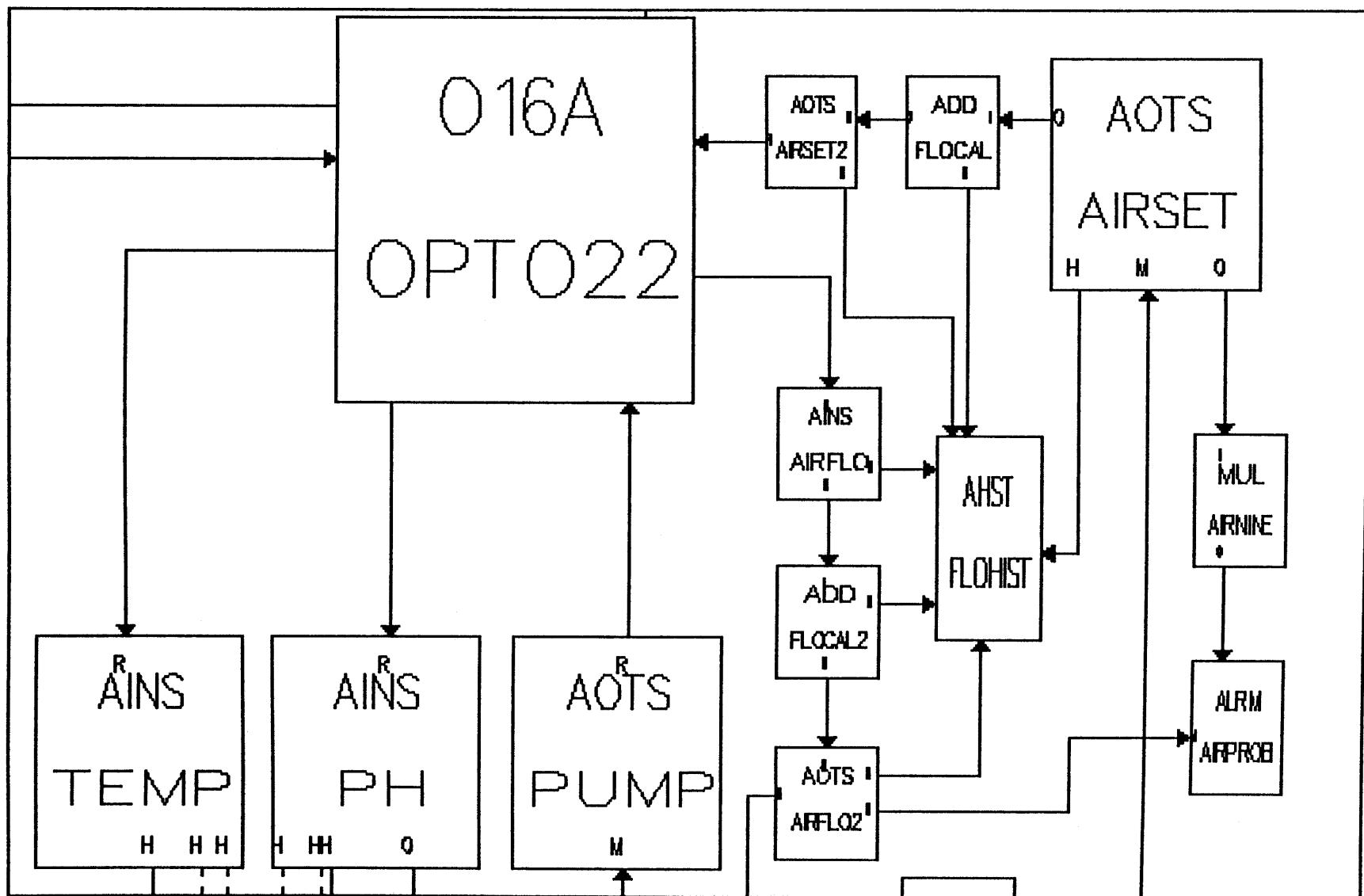


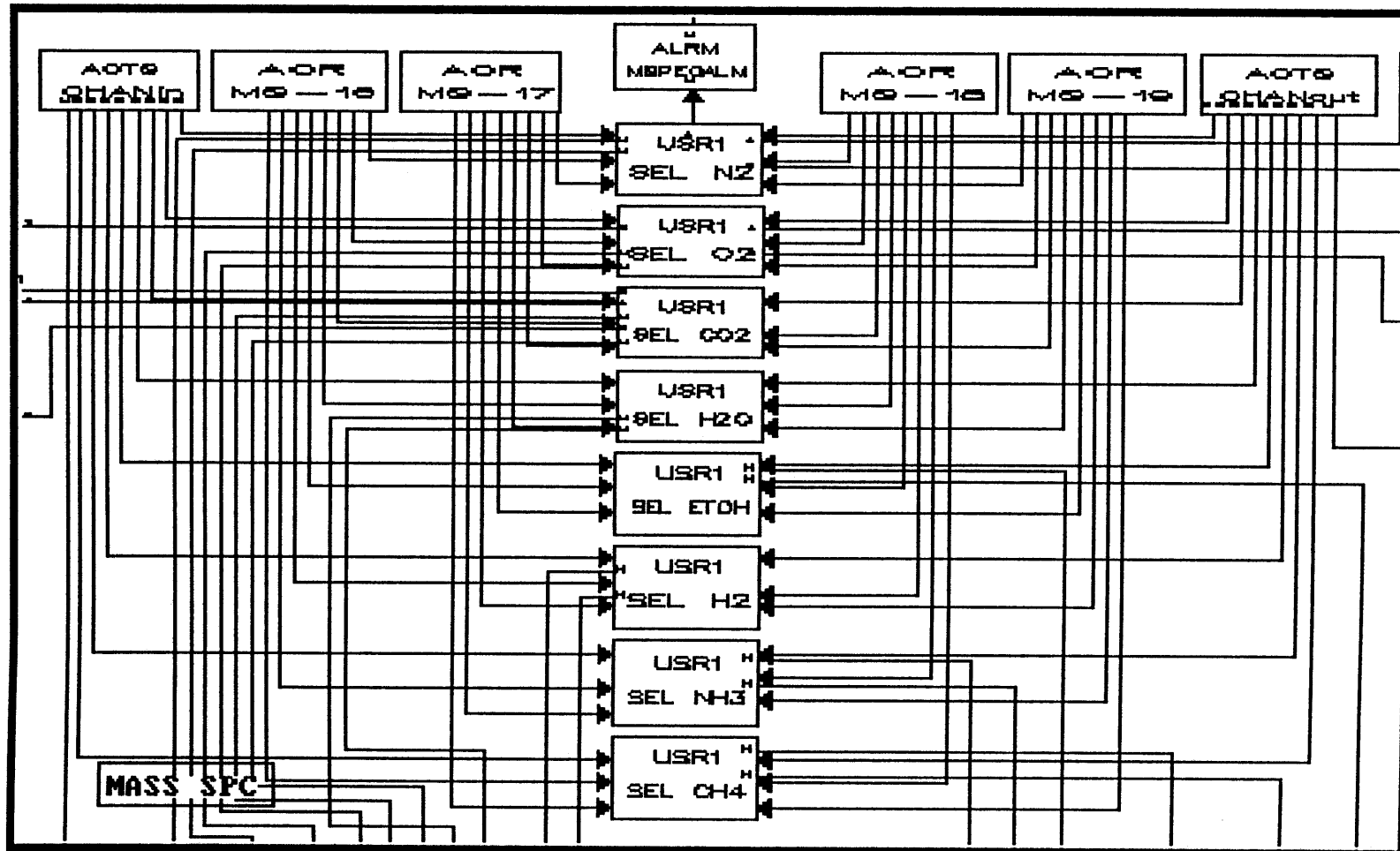


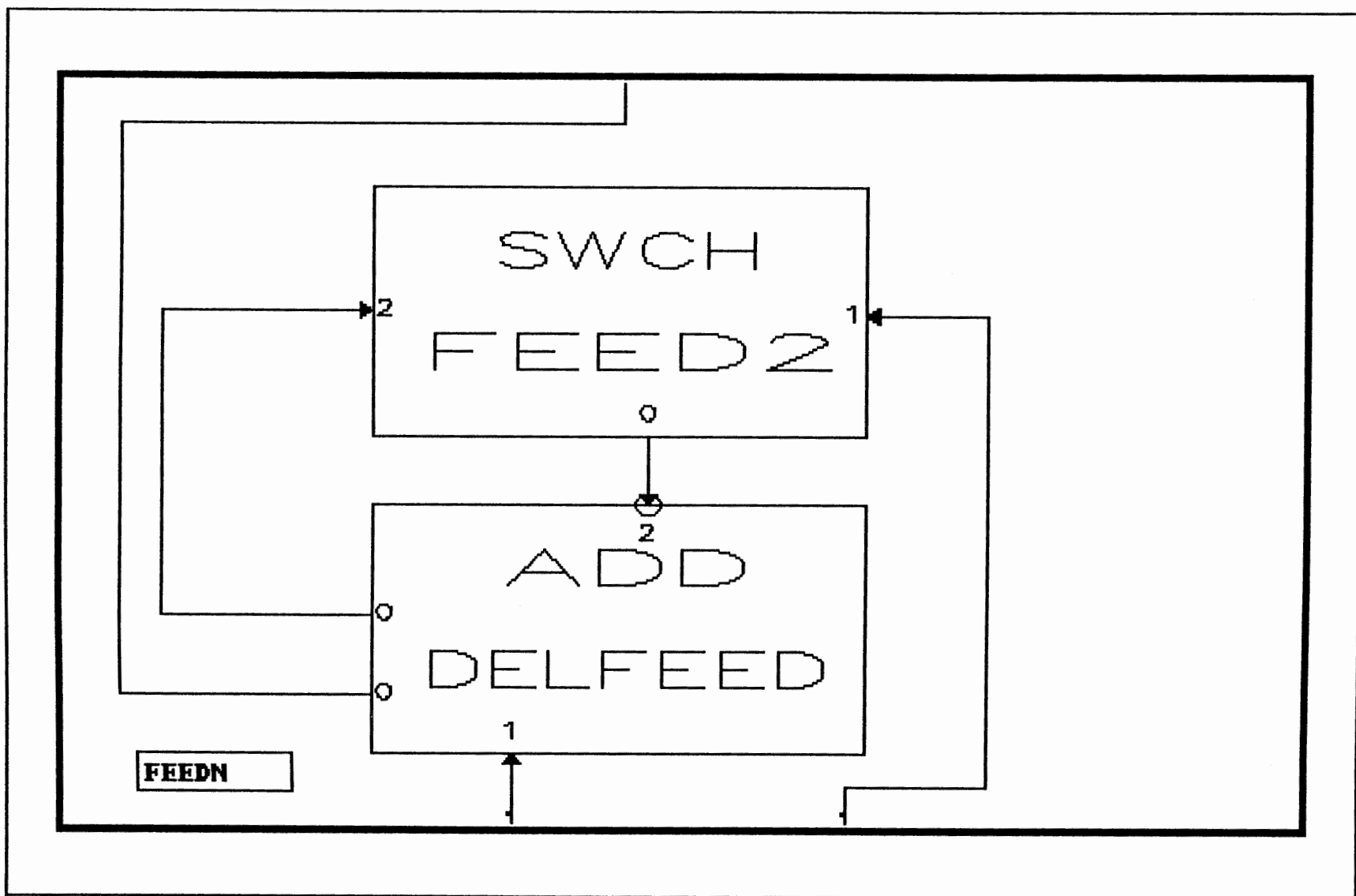






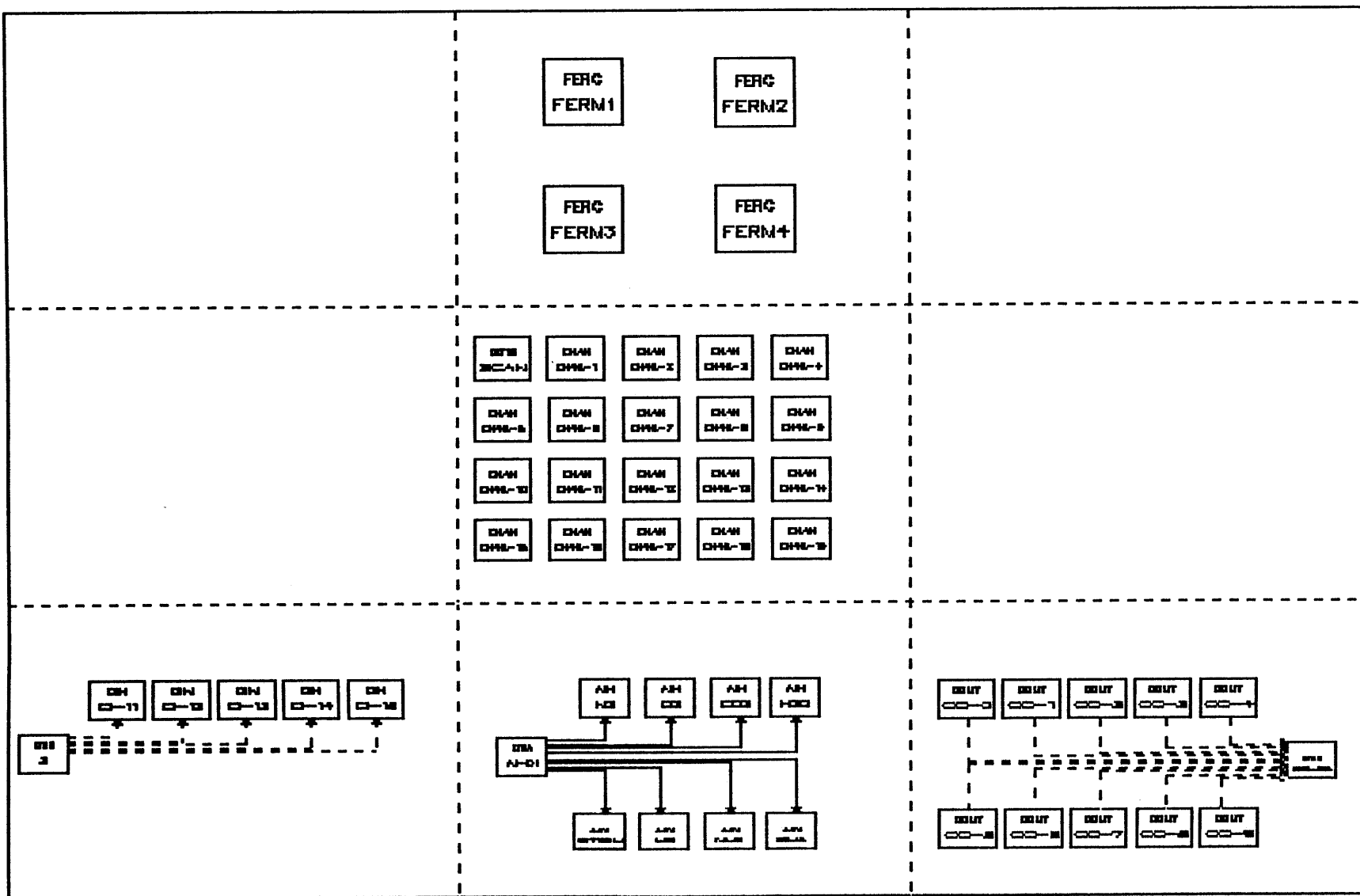






Next is the Paragon database that controls the mass spectrometer. This database was constructed by Intec Controls Corporation. The following detail is shown:

- 1) The entire database containing five screens.
- 2) An enlargement of screens 1-5.
- 3) Detail inside blocks FERM1 and CHANL-10



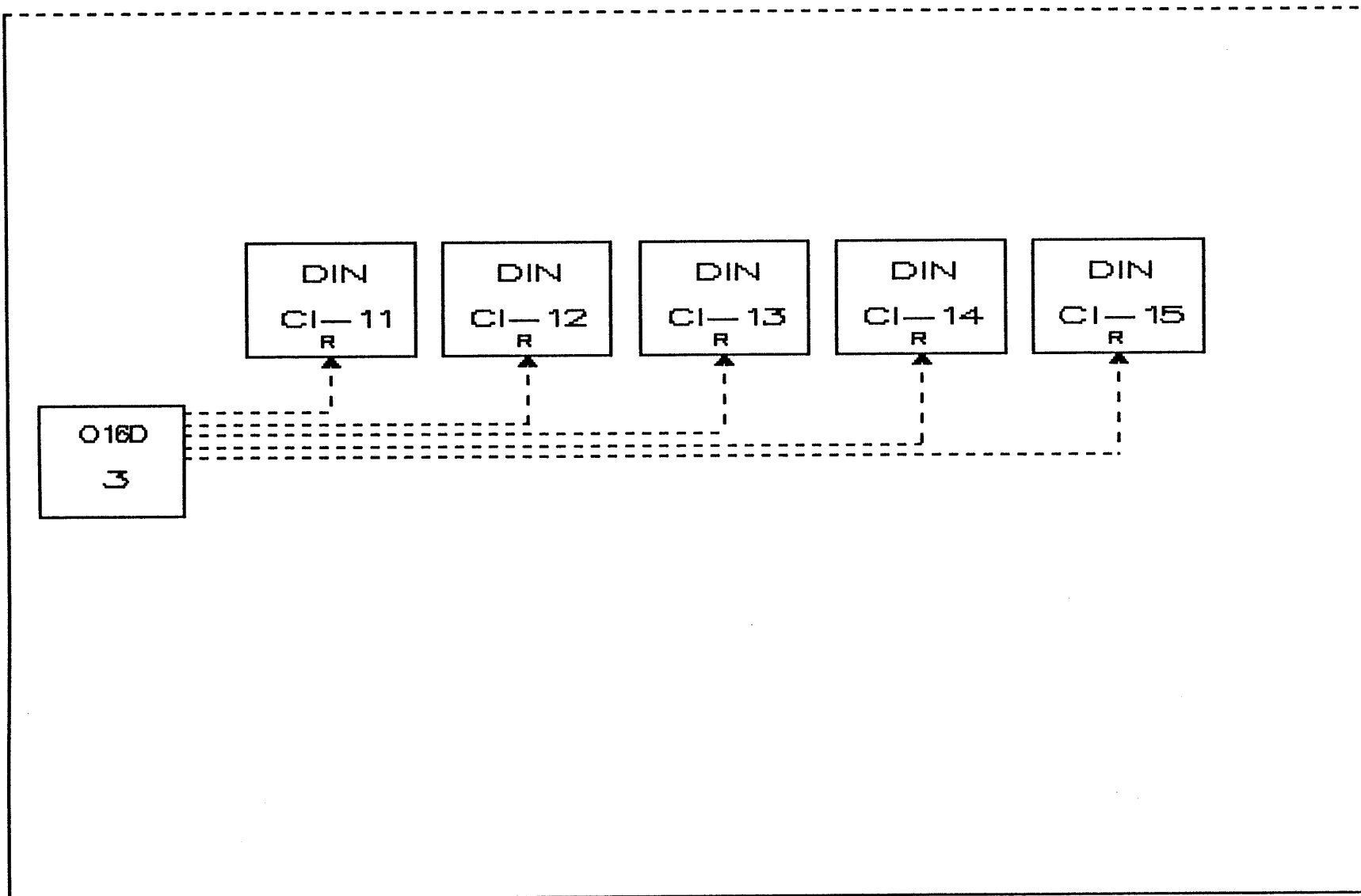
FERC
FERM1

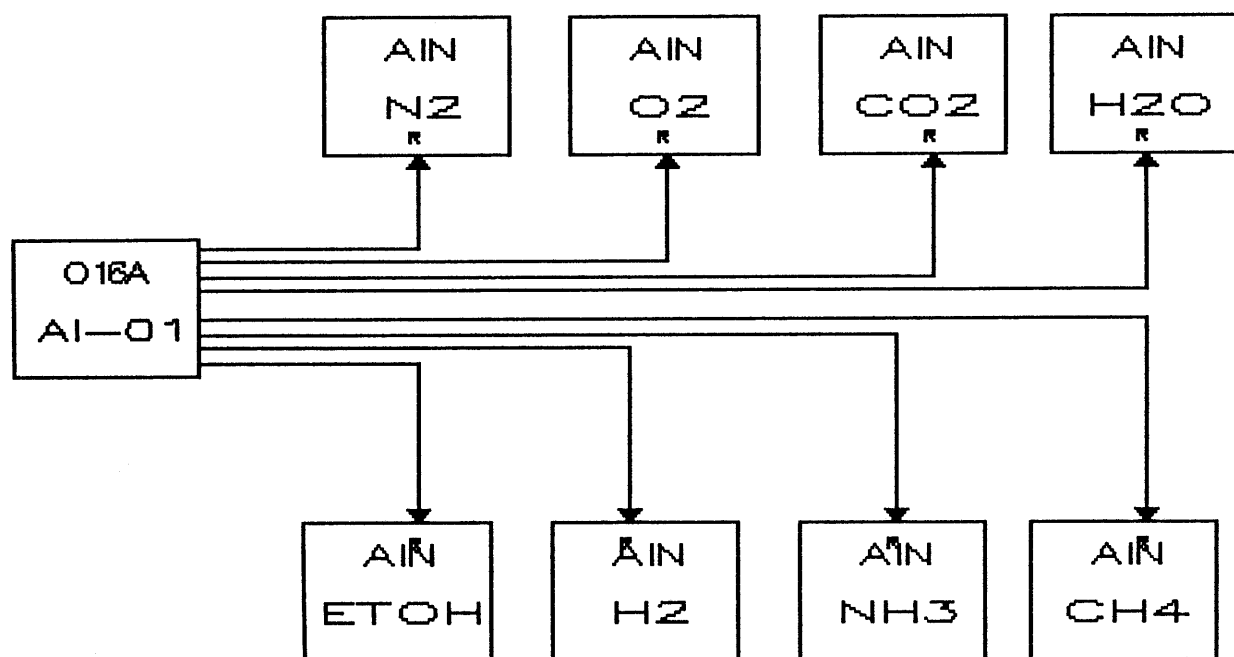
FERC
FERM2

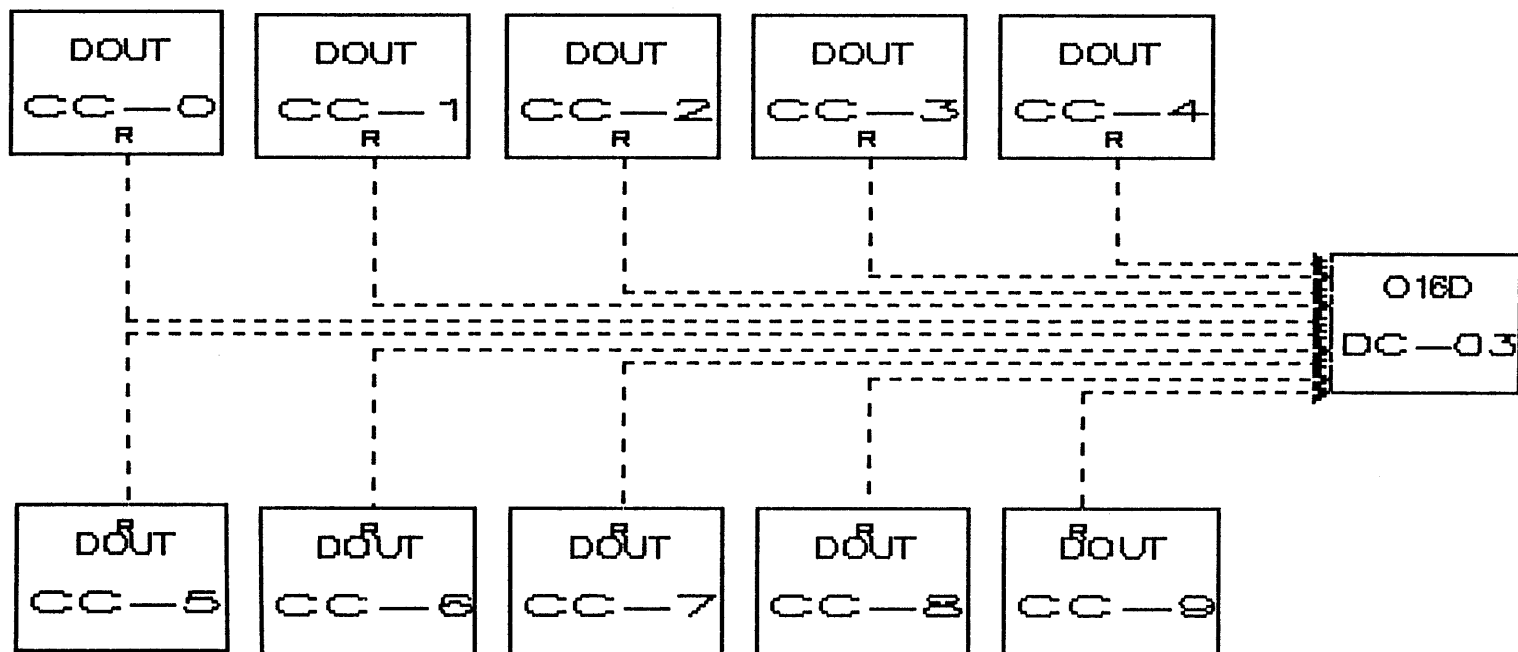
FERC
FERM3

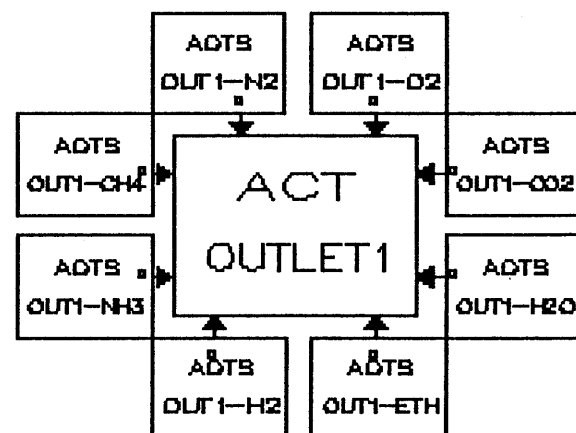
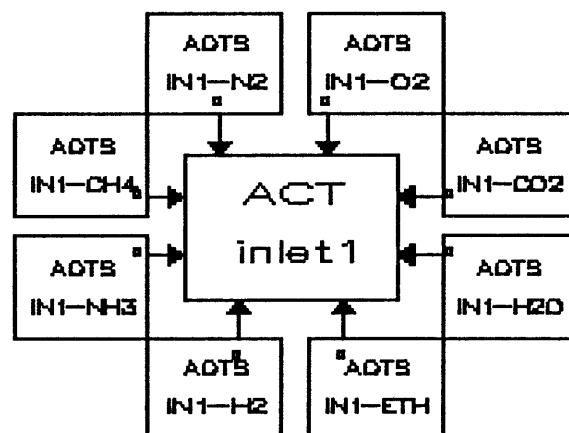
FERC
FERM4

DOTS SCAN	CHAN CHANL-1	CHAN CHANL-2	CHAN CHANL-3	CHAN CHANL-4
CHAN CHANL-5	CHAN CHANL-6	CHAN CHANL-7	CHAN CHANL-8	CHAN CHANL-9
CHAN CHANL-10	CHAN CHANL-11	CHAN CHANL-12	CHAN CHANL-13	CHAN CHANL-14
CHAN CHANL-15	CHAN CHANL-16	CHAN CHANL-17	CHAN CHANL-18	CHAN CHANL-19





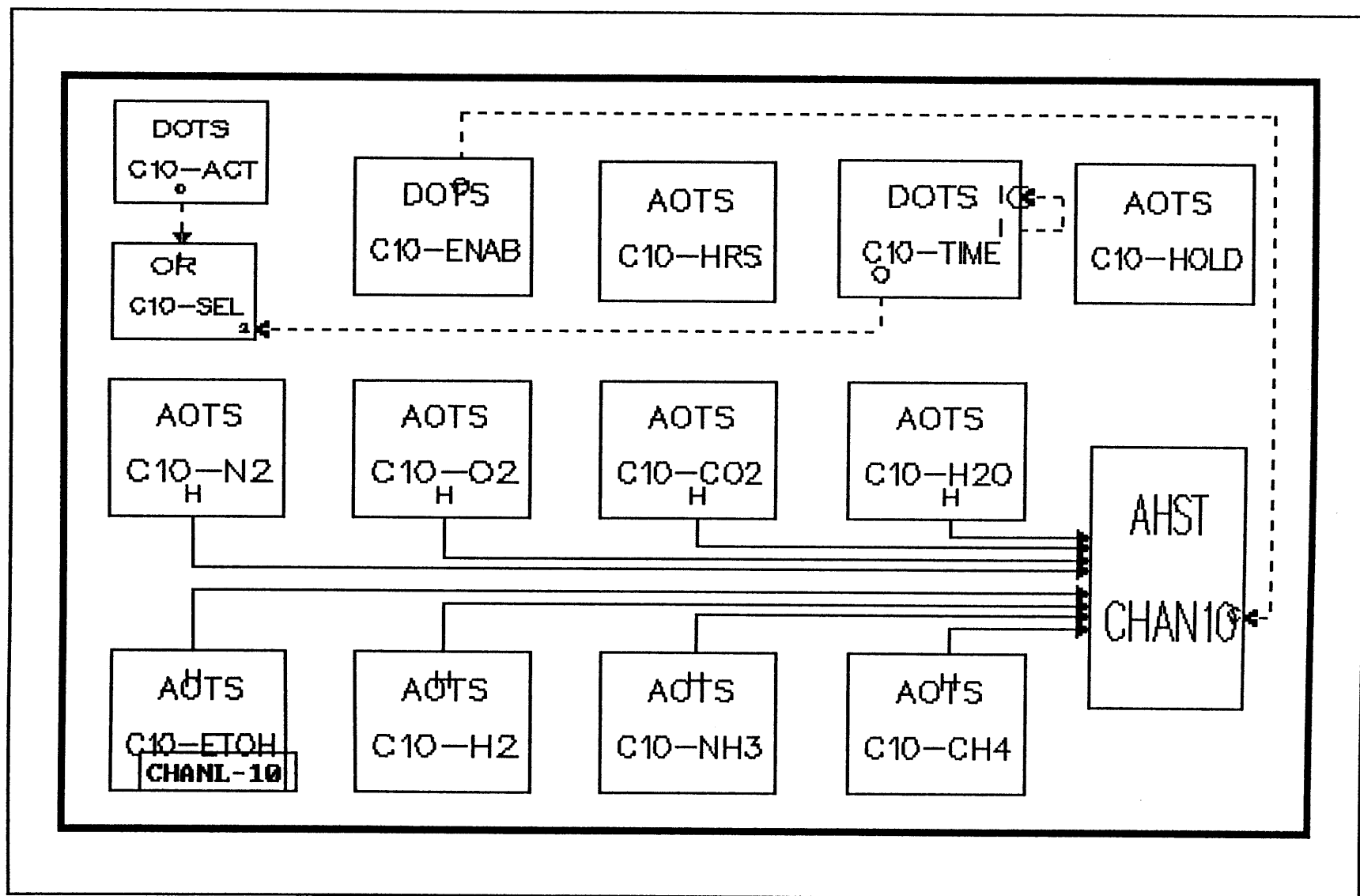




AOTS
IN1CH

AOTS
OUT1CH

FERM1



The C subroutines developed were as follows:

- USER1.C This subroutine selects the mass spectrometer channel number from which to read the data.
- USER2.C This subroutine calculates the elapsed time.
- USER3.C This subroutine determines the glucose concentration based on a Monod model and a measured growth rate (assumes fermentation is glucose limited).
- USER4.C This subroutine predicts the dissolved fishmeal concentration and the amount of fishmeal to add based on an optimized model.
- USER5.C This subroutine determines the dissolved fishmeal concentration based on a Monod model and a measured growth rate (assumes fermentation is dissolved fishmeal limited).
- USER6.C This subroutine determines when to add the fishmeal.
- USER_C1.C This subroutine configures communication port 1 to accept data from the Ohaus multiplexer. It enables three balances to be read through one communication port. This program was developed in the most part by J. Hong of Ohaus Corporation.

```

/*****
/* $Revision: 1.0 $   Intec Controls Corp.   $Date: 29 Dec 1988 13:56:34 $*/
/*****
/*
/* This work contains confidential and proprietary trade secrets of
/* Intec Controls Corp.  Reproduction, disclosure or use without specific
/* authorization of Intec Controls Corp. is prohibited.
/*
/*
/*****
/*
/* Copyright(C) by Intec Controls Corp, 1986, 1987, 1988
/* All rights reserved: Intec Control Corp. 1988
/*
/*****
/*
/* File Name      : USER1.C
/* Date           : 12/1/88
/*
/* Description    : Contains the User Block #1 Algorithm Routine
/*
/* Functions included:   user_block1()
/*
/* REVISION HISTORY :
/* $Log: C:/runtime/comm/user/vcs/user1.c_v $
*
* Rev 1.0 29 Dec 1988 13:56:34 unknown
* Initial revision.
*
* Rev 1.0 12 Dec 1988 11:58:02 unknown
* Initial revision.
*/
/*
/*
/*****

```

```

#include <stdio.h>
#include "user.h"

```

```

/*****
*          user_block1(blk_ptr)
*
* Description : USER Block #1 Algorithm Routine.
*
*****
user_block1(blk_ptr)
USER *blk_ptr
;

{
    float ain[8],chin,chsix,chsev,cheig,chnin,sel,chout,selt;
    float delta;

```

```

get_analog_inputs(blk_ptr,ain);

chin = ain[0];
chsix = ain[1];
chsev = ain[2];
cheig = ain[3];
chnin = ain[4];
chout = ain[5];

delta = 0.001;
sel = 0.0;
selt = 0.0;

if (chin > (16.0-delta) && chin < (16.0+delta))
    sel=chsix;
if (chin > (17.0-delta) && chin < (17.0+delta))
    sel=chsev;
if (chin > (18.0-delta) && chin < (18.0+delta))
    sel=cheig;
if (chin > (19.0-delta) && chin < (19.0+delta))
    sel=chnin;

if (chout > (16.0-delta) && chout < (16.0+delta))
    selt=chsix;
if (chout > (17.0-delta) && chout < (17.0+delta))
    selt=chsev;
if (chout > (18.0-delta) && chout < (18.0+delta))
    selt=cheig;
if (chout > (19.0-delta) && chout < (19.0+delta))
    selt=chnin;

blk_ptr->ch_a=sel;
blk_ptr->ch_b=selt;

}

/*----- End of USER1.C -----*/

```

```

/*****
/* $Revision: 1.0 $   Intec Controls Corp.   $Date: 29 Dec 1988 13:56:36 $*/
/*****
/*
/* This work contains confidential and proprietary trade secrets of
/* Intec Controls Corp.  Reproduction, disclosure or use without specific
/* authorization of Intec Controls Corp. is prohibited.
/*
/*
/*****
/*
/* Copyright(C) by Intec Controls Corp, 1986, 1987, 1988
/* All rights reserved: Intec Control Corp. 1988
/*
/*
/*****
/*
/* File Name      : USER2.C
/* Date           : 12/1/88
/*
/* Description    : Contains the User Block #2 Algorithm Routine
/*
/* Functions included:   user_block2()
/*
/* REVISION HISTORY :
/* $Log: C:/runtime/comm/user/vcs/user2.c_v $
*
* Rev 1.0  29 Dec 1988 13:56:36  unknown
* Initial revision.
*
* Rev 1.0  12 Dec 1988 11:58:02  unknown
* Initial revision.
*/
*/
*/
/*****/

```

```

#include <stdio.h>
#include "user.h"

```

```

/*****
*           user_block2(blk_ptr)
*
* Description : USER Block #2 Algorithm Routine.
*
*****/
user_block2(blk_ptr)
USER *blk_ptr
;

{
    float   ain[5],iday,rday,etime;
    int     day,month,year,weekday,mnthn,inocyr,inocmn,inocdy;
    int     inochr;

```

```

int    hour,mnths;
int    inocmi,inocse,minute,second;
float  finocmi,finocse,fminute,fsecond;

get_analog_inputs(blk_ptr,ain);
get_date(&day,&month,&year,&weekday);
get_time(&hour,&minute,&second);

```

```

if (month == 1)
    mnths=0;
if (month == 2)
    mnths=31;
if (month == 3)
    mnths=59;
if (month == 4)
    mnths=90;
if (month == 5)
    mnths=120;
if (month == 6)
    mnths=151;
if (month == 7)
    mnths=181;
if (month == 8)
    mnths=212;
if (month == 9)
    mnths=243;
if (month == 10)
    mnths=273;
if (month == 11)
    mnths=304;
if (month == 12)
    mnths=334;

```

```

blk_ptr->ch_a=year;
blk_ptr->ch_b=month;
blk_ptr->ch_c=day;

```

```

inocyr = ain[0];
inocmn = ain[1];
inocdy = ain[2];
inochr = ain[3];
inocmi = ain[4];
inocse = ain[5];

```

```

if (inocmn == 1)
    mnths=0;
if (inocmn == 2)
    mnths=31;
if (inocmn == 3)
    mnths=59;

```



```

if (inocmn == 4)
    mnthi = 90;
if (inocmn == 5)
    mnthi = 120;
if (inocmn == 6)
    mnthi = 151;
if (inocmn == 7)
    mnthi = 181;
if (inocmn == 8)
    mnthi = 212;
if (inocmn == 9)
    mnthi = 243;
if (inocmn == 10)
    mnthi = 273;
if (inocmn == 11)
    mnthi = 304;
if (inocmn == 12)
    mnthi = 334;

```

```

iday = mnthi + inocdy;
rday = mnthn + day;
finocmi = inocmi;
finocse = inocse;
fminute = minute;
fsecond = second;

```

```
etime = (year - inocyr) * 8760 + (rday - iday) * 24 + (hour - inochr) + (fminute - finocmi) / 60 + (fsecond - finocse) / 3600;
```

```
blk_ptr->ch_d = etime;
```

```
}
```

```
/*----- End of USER2.C -----*/
```

```

/*****
/* $Revision: 1.0 $   Intec Controls Corp.   $Date: 29 Dec 1988 13:56:38 $*/
/*****
/*                                     */
/* This work contains confidential and proprietary trade secrets of       */
/* Intec Controls Corp.  Reproduction, disclosure or use without specific   */
/* authorization of Intec Controls Corp. is prohibited.                     */
/*                                     */
/*****
/*                                     */
/* Copyright(C) by Intec Controls Corp, 1986, 1987, 1988                   */
/* All rights reserved: Intec Control Corp. 1988                           */
/*                                     */
/*****
/*                                     */
/* File Name      : USER3.C                                                */
/* Date           : 12/1/88                                                */
/*                                     */
/* Description    : Contains the User Block #3 Algorithm Routine           */
/*                                     */
/* Functions included:   user_block3()                                     */
/*                                     */
/* REVISION HISTORY :                                                       */
/* $Log: C:/runtime/comm/user/vcs/user3.c_v $                               */
*
* Rev 1.0 29 Dec 1988 13:56:38 unknown
* Initial revision.
*
* Rev 1.0 12 Dec 1988 11:58:04 unknown
* Initial revision.
*/
*/
*/
/*****/

```

```

#include <stdio.h>
#include "user.h"

```

```

/*****
*          user_block3(blk_ptr)          *
*                                     *
* Description : USER Block #3 Algorithm Routine.      *
*                                     *
*****/
user_block3(blk_ptr)
USER *blk_ptr      ;

{
    float ain[8],mu,over,gluc,testmu,glucs;

    get_analog_inputs(blk_ptr,ain);

```

```
mu=ain[0];
over=ain[1];

gluc=0.0;
back: testmu=0.91*gluc/(4.1+gluc);

if ( testmu < mu )
    gluc=gluc+0.05;
if ( testmu < mu )
    goto back;

glucs=gluc+over;

blk_ptr->ch_a=gluc;
blk_ptr->ch_b=glucs;

}

/*----- End of USER3.C -----*/
```

```

/*****
/* $Revision: 1.0 $ Intec Controls Corp. $Date: 29 Dec 1988 13:56:40 $ */
/*****
/*
/* This work contains confidential and proprietary trade secrets of
/* Intec Controls Corp. Reproduction, disclosure or use without specific
/* authorization of Intec Controls Corp. is prohibited.
/*
/*****
/*
/* Copyright(C) by Intec Controls Corp, 1986, 1987, 1988
/* All rights reserved: Intec Control Corp. 1988
/*
/*****
/*
/* File Name : USER4.C
/* Date : 12/1/88
/*
/* Description : Contains the User Block #4 Algorithm Routine
/*
/* Functions included: user_block4()
/*
/* REVISION HISTORY :
/* $Log: C:/runtime/comm/user/vcs/user4.c_v $
*
* Rev 1.0 29 Dec 1988 13:56:40 unknown
* Initial revision.
*
* Rev 1.0 12 Dec 1988 11:58:06 unknown
* Initial revision.
*/
/*
/*
/*****

```

```

#include <stdio.h>
#include "user.h"

```

```

/*****
*
* user_block4(blk_ptr)
*
* Description : USER Block #4 Algorithm Routine.
*
*****/
user_block4(blk_ptr)
USER *blk_ptr ;

{
    float ain[8],elapsed,cogas,coset,feed,star,tgrow,diff,d;

    get_analog_inputs(blk_ptr,ain);

```

```

elapse=ain[0];
cogas=ain[1];
coset=ain[2];
star=ain[3];
tgrow=ain[4];

if(cogas > coset)
    star+ +;

blk_ptr->ch_a=star;

if(star==1)
    tgrow=elapse;

diff=elapse-tgrow;

feed=0;
d=0;

if(star>=1 && diff<8.125)
    feed=0;
if(star>=1 && diff>=0.0 && diff<0.125)
    d=0.50;
if(star>=1 && diff>=0.125 && diff<0.375)
    d=0.59;
if(star>=1 && diff>=0.375 && diff<0.625)
    d=0.82;
if(star>=1 && diff>=0.625 && diff<0.875)
    d=1.07;
if(star>=1 && diff>=0.875 && diff<1.125)
    d=1.33;
if(star>=1 && diff>=1.125 && diff<1.375)
    d=1.60;
if(star>=1 && diff>=1.375 && diff<1.625)
    d=1.88;
if(star>=1 && diff>=1.625 && diff<1.875)
    d=2.18;
if(star>=1 && diff>=1.875 && diff<2.125)
    d=2.48;
if(star>=1 && diff>=2.125 && diff<2.375)
    d=2.78;
if(star>=1 && diff>=2.375 && diff<2.625)
    d=3.09;
if(star>=1 && diff>=2.625 && diff<2.875)
    d=3.39;
if(star>=1 && diff>=2.875 && diff<3.125)
    d=3.68;
if(star>=1 && diff>=3.125 && diff<3.375)
    d=3.96;
if(star>=1 && diff>=3.375 && diff<3.625)
    d=4.23;

```

```

if(star>=1 && diff>=3.625 && diff<3.875)
    d=4.48;
if(star>=1 && diff>=3.875 && diff<4.125)
    d=4.71;
if(star>=1 && diff>=4.125 && diff<4.375)
    d=4.92;
if(star>=1 && diff>=4.375 && diff<4.625)
    d=5.10;
if(star>=1 && diff>=4.625 && diff<4.875)
    d=5.26;
if(star>=1 && diff>=4.875 && diff<5.125)
    d=5.39;
if(star>=1 && diff>=5.125 && diff<5.375)
    d=5.49;
if(star>=1 && diff>=5.375 && diff<5.625)
    d=5.55;
if(star>=1 && diff>=5.625 && diff<5.875)
    d=5.58;
if(star>=1 && diff>=5.875 && diff<6.125)
    d=5.58;
if(star>=1 && diff>=6.125 && diff<6.375)
    d=5.53;
if(star>=1 && diff>=6.375 && diff<6.625)
    d=5.44;
if(star>=1 && diff>=6.625 && diff<6.875)
    d=5.30;
if(star>=1 && diff>=6.875 && diff<7.125)
    d=5.12;
if(star>=1 && diff>=7.125 && diff<7.375)
    d=4.88;
if(star>=1 && diff>=7.375 && diff<7.625)
    d=4.59;
if(star>=1 && diff>=7.625 && diff<7.875)
    d=4.25;
if(star>=1 && diff>=7.875 && diff<8.125)
    d=3.86;
if(star>=1 && diff>=8.125 && diff<8.375)
{ d=3.42;
  feed=2.8; }
if(star>=1 && diff>=8.375 && diff<8.625)
{ d=3.19;
  feed=9.1; }
if(star>=1 && diff>=8.625 && diff<8.875)
{ d=3.09;
  feed=6.0; }
if(star>=1 && diff>=8.875 && diff<9.125)
{ d=3.00;
  feed=3.5; }
if(star>=1 && diff>=9.125 && diff<9.375)
{ d=2.91;
  feed=1.8; }

```

```

if(star>=1 && diff>=9.375 && diff<9.625)
{ d=2.82;
  feed=1.1; }
if(star>=1 && diff>=9.625 && diff<9.875)
{ d=2.73;
  feed=1.0; }
if(star>=1 && diff>=9.875 && diff<10.125)
{ d=2.65;
  feed=1.4; }
if(star>=1 && diff>=10.125 && diff<10.375)
{ d=2.57;
  feed=2.1; }
if(star>=1 && diff>=10.375 && diff<10.625)
{ d=2.49;
  feed=2.8; }
if(star>=1 && diff>=10.625 && diff<10.875)
{ d=2.41;
  feed=3.4; }
if(star>=1 && diff>=10.875 && diff<11.125)
{ d=2.34;
  feed=3.9; }
if(star>=1 && diff>=11.125 && diff<11.375)
{ d=2.27;
  feed=4.1; }
if(star>=1 && diff>=11.375 && diff<11.625)
{ d=2.20;
  feed=4.2; }
if(star>=1 && diff>=11.625 && diff<11.875)
{ d=2.13;
  feed=4.2; }
if(star>=1 && diff>=11.875 && diff<12.125)
{ d=2.07;
  feed=5.4; }
if(star>=1 && diff>=12.125 && diff<12.375)
  feed=10.4;
if(star>=1 && diff>=12.375 && diff<12.625)
  feed=4.1;
if(star>=1 && diff>=12.625 && diff<12.875)
  feed=4.1;
if(star>=1 && diff>=12.875 && diff<13.125)
  feed=11.2;
if(star>=1 && diff>=13.125 && diff<13.375)
  feed=5.1;
if(star>=1 && diff>=13.375 && diff<13.625)
  feed=10.7;
if(star>=1 && diff>=13.625 && diff<13.875)
  feed=7.6;
if(star>=1 && diff>=13.875 && diff<14.125)
  feed=10.8;
if(star>=1 && diff>=14.125 && diff<14.375)
  feed=9.7;

```

```

if(star>=1 && diff>=14.375 && diff<14.625)
    feed=11.6;
if(star>=1 && diff>=14.625 && diff<14.875)
    feed=11.6;
if(star>=1 && diff>=14.875 && diff<15.125)
    feed=12.9;
if(star>=1 && diff>=15.125 && diff<15.375)
    feed=13.6;
if(star>=1 && diff>=15.375 && diff<15.625)
    feed=14.7;
if(star>=1 && diff>=15.625 && diff<15.875)
    feed=15.6;
if(star>=1 && diff>=15.875 && diff<16.125)
    feed=16.8;
if(star>=1 && diff>=16.125 && diff<16.375)
    feed=18.0;
if(star>=1 && diff>=16.375 && diff<16.625)
    feed=19.3;
if(star>=1 && diff>=16.625 && diff<16.875)
    feed=20.6;
if(star>=1 && diff>=16.875 && diff<17.125)
    feed=22.1;
if(star>=1 && diff>=17.125 && diff<17.375)
    feed=23.7;
if(star>=1 && diff>=17.375 && diff<17.625)
    feed=25.4;
if(star>=1 && diff>=17.625 && diff<17.875)
    feed=27.3;
if(star>=1 && diff>=17.875 && diff<18.125)
    feed=29.3;
if(star>=1 && diff>=18.125)
    feed=31.4;
if(star>=1 && diff>=12.125)
    d=2.05;

blk_ptr->ch_b=lgrow;
blk_ptr->ch_c=feed;
blk_ptr->ch_d=d;
}

/*----- End of USER4.C -----*/

```



```

/*****
/* $Revision: 1.0 $   Intec Controls Corp.   $Date: 29 Dec 1988 13:56:40 $*/
/*****
/*
/* This work contains confidential and proprietary trade secrets of
/* Intec Controls Corp.  Reproduction, disclosure or use without specific
/* authorization of Intec Controls Corp. is prohibited.
/*
/*
/*****
/*
/* Copyright(C) by Intec Controls Corp, 1986, 1987, 1988
/* All rights reserved: Intec Control Corp. 1988
/*
/*
/*****
/*
/* File Name      : USER5.C
/* Date           : 12/1/88
/*
/* Description    : Contains the User Block #5 Algorithm Routine
/*
/* Functions included:   user_block5()
/*
/* REVISION HISTORY :
/* $Log: C:/runtime/comm/user/vcs/user5.c_v $
*
* Rev 1.0 29 Dec 1988 13:56:40 unknown
* Initial revision.
*
* Rev 1.0 12 Dec 1988 11:58:06 unknown
* Initial revision.
*/
/*
/*
/*****

```

```

#include <stdio.h>
#include "user.h"

```

```

/*****
*          user_block5(blk_ptr)
*
* Description : USER Block #5 Algorithm Routine.
*
*****/
user_block5(blk_ptr)
USER *blk_ptr
;

{
    float ain[8],mud,dd,test;

    get_analog_inputs(blk_ptr,ain);

```

```
mud=ain[0];

dd=0;
backy: test=0.91*dd/(3.9+dd);

if( test<mud )
    dd=dd+0.02;

if( test<mud )
    goto backy;

blk_ptr->ch_a=dd;

}

/*----- End of USER5.C -----*/
```

```

/*****
/* $Revision: 1.0 $   Intec Controls Corp.   $Date: 29 Dec 1988 13:56:42 $*/
/*****
/*                                     */
/* This work contains confidential and proprietary trade secrets of          */
/* Intec Controls Corp.  Reproduction, disclosure or use without specific      */
/* authorization of Intec Controls Corp. is prohibited.                       */
/*                                     */
/*****
/*                                     */
/* Copyright(C) by Intec Controls Corp, 1986, 1987, 1988                     */
/* All rights reserved: Intec Control Corp. 1988                             */
/*                                     */
/*****
/*                                     */
/*   File Name       : USER6.C                                               */
/*   Date            : 12/1/88                                                */
/*                                     */
/*   Description      : Contains the User Block #6 Algorithm Routine          */
/*                                     */
/*   Functions included:   user_block6()                                     */
/*                                     */
/*   REVISION HISTORY :                                                       */
/*   $Log: C:/runtime/comm/user/vcs/user6.c_v $                               */
*
*   Rev 1.0  29 Dec 1988 13:56:42   unknown
*   Initial revision.
*
*   Rev 1.0  12 Dec 1988 11:58:08   unknown
*   Initial revision.
*/
/*                                     */
/*                                     */
/*****

```

```

#include <stdio.h>
#include "user.h"

```

```

/*****
*           user_block6(blk_ptr)                                           *
*                                                                 *
*   Description : USER Block #6 Algorithm Routine.                      *
*                                                                 *
*****/
user_block6(blk_ptr)
USER *blk_ptr      ;

{
    float ain[8],fish,fisha,count,max1,max2,shoot,add,icount;

    get_analog_inputs(blk_ptr,ain);

```

```
fish=ain[0];
fisha=ain[1];
max1=ain[2];
max2=ain[3];
count=ain[4];

shoot=0;
add=1;
icount=count;

if(fish>0)
    max1=1.0;
if(fisha>0)
    max2=1.0;
if(max1==1 && max2==1)
    shoot=1;
if(max2==1)
    count=icount+add;
if(count==174)
{max1=0;
max2=0;
count=0;}

blk_ptr->ch_a=max1;
blk_ptr->ch_b=max2;
blk_ptr->ch_c=shoot;
blk_ptr->ch_d=count;

}

/*----- End of USER6.C -----*/
```

PROGRAM USER_C1.C

```

/*
 * OHAUS.C - file of C routine compiled into Paragon to accept
 *          mux'ed scale readings during Paragon Operatorion.
 *          Programmed 6/19/89  by Joseph M. Hong
 */

```

```

#include <string.h>
#include <math.h>
#include "user.h"

```

```

#define BUFSIZE 80

```

```

char string[BUFSIZE];
char command;
char seom[4] = { 0,0,13,0 } ;

```

```

user_comm1(ptr)
struct USER_DEF *ptr;
{
    static int i;
    char buf[BUFSIZE];

    send_mux(ptr);
}

```

```

#define PORT_ID_STR_LEN 2

```

```

rcv_user_comm1(ptr,msg_num,msg,len,error)
struct USER_DEF *ptr;
char msg[BUFSIZE];
int msg_num,len,error;
{

```

```

    char *msg_ptr;
    int i,port = 0;
    float wt = 0.0;
    char dum_buf[BUFSIZE];

```

```

    if ( (error == 0) && (len != 0) )
    {
        print_usermsg(msg);
        msg_ptr = msg;
        /*

```

```

        sscanf(msg,"%d%f",&port,&wt);
        */
        for ( i = 0 ; i < PORT_ID_STR_LEN ; i++ ) dum_buf[i] = *msg_ptr++;
        dum_buf[i] = '\0';
        wt = atof(msg_ptr);
        port = atoi(dum_buf);
        if ( port > 4 || port < 1 ) port = 0;

        switch ( port ) {
        case 1 : ptr->ch_a = wt;
                break;
        case 2 : ptr->ch_b = wt;
                break;
        case 3 : ptr->ch_c = wt;
                break;
        case 4: ptr->ch_d = wt;
                break;
        default: break;
        }
    }
    return(1);
}

#define CR 13
#define LF 10
#define CAPITAL_T 20
#define TIMEOUT 100
#define REPEAT_CYCLES 5

send_mux(ptr)
struct USER_DEF *ptr;
{

    char  msg[BUFSIZE],*msg_ptr;
    static int port_num;

    msg_ptr = msg;

    if ( port_num > 3 ) port_num = 0 ;

    port_num++;

    sprintf(msg,"%c%dP%c%c",CAPITAL_T,port_num,CR,LF);

    send_usermsg(ptr,1,msg,strlen(msg),REPEAT_CYCLES,TIMEOUT,BUFSIZE,seom,0);
}

```

APPENDIX C

Signal Conditioning Unit for the PC Based Data Acquisition and Control System

The signal conditioning unit for the PC-based data acquisition system was based on various components which were purchased separately and assembled by Roy Brewer with the aid of Charles Paton, Steven Lee, and Max Kennedy. The components were as follows:

Components from Opto 22, 15461 Springdale St., Huntington Beach, CA 92649

- 1) 16 Slot Analog I/O Rack (PB16AH).
- 2) 16 Slot Digital I/O Rack (PB16HC).
- 3) Analog Brain Board (B2).
- 4) Digital Brain Board (B1).
- 5) RS-422/485 Adapter Board (AC422).
- 6) 5-60 volt DC Output Modules (ODC5) x2.
- 7) 0-5 volt DC Input Module (AD6) x5.
- 8) 0-100 mV Input Module (AD13T) x2.
- 9) 0-5 volt DC Output Module (DA4) x2.

Component from Eagle Electric Company Inc., 195 Old Colony Avenue, Boston, MA 02127.

- 1) Hoffman Metal Box Type 1 Enclosure.

Component from Lambda Electronics, 515 Broad Hollow Rd., Melville, Long Island, NY 11747.

- 1) Industrial Power Supply, Triple Outlet (LST-37-144).

APPENDIX D

Fluorescence Spectroscopy V 1.1

Used to Control the Fluorimeter During Light Scatter Experiments

```

100 REM FLUORESCENCE (VERSION 1.1)
110 REM HOMBREW VERSION OF VERSION 1.0
120 REM ORIGINAL:
122 REM   COPYRIGHT 1984 BY ORIEL CORP.
124 REM   AUTHORS T.C.O'HAVER & J.W.RAFFERTY
126 REM UPDATE:
128 REM   COMPLETED AUGUST, 1987
130 REM   BY PETER SULLIVAN, MIT
140 REM   *** HOUSEKEEPING TASKS ***
155 DIM RE(10)
180 ST$ = "-----"
190 SK$ = "===== "
195 SC$ = " EXCITATION "
196 TD = 1000
197 L$ = "INTENSITY (NANOAMPS)":BL$ = "          ":F$ = " "
200 GA = 1:SL = 2:CL = 5:PL = 0:RT = .1:NR = 20:D = 1:EG$ = "1200":FG$ = EG$
202 LT$ = "XENON":DT$ = "PM #7062":EF$ = "NONE":FF$ = EF$:ES$ = "2 NM":FS$ = ES$
205 S1 = 0:S2 = 50:EI = 1:FI = 1:OF = 0:NB = 1:MO$ = " NON-RATIO "
210 D$ = CHR$(4):AD = - 16256 + 16 * SL
220 DIM BA$(50): LET BA$(0) = "USER": LET BA = 0: LET BA$ = " "
230 LO = AD:HI = AD + 1:TF = 256:SK = AD + 6
231 DIM DC(2),GM(2):DC(1) = 0:DC(2) = 0:GM(1) = 6:GM(2) = 6
232 FOR I = 0 TO 6:GP(I) = I: NEXT I
234 REM   READ IN SCALING FACTORS
235 FOR I = 0 TO 6: READ MF(I): NEXT I
236 DATA   999630,99963,9996.3,99          9.63,99.963,9.9963,.99963
238 SP(1) = 0:SP(2) = 0
242 G1 = GA:G2 = G1:M1 = MF(G1):M2 = MF(G2)
243 FOR I = 768 TO 777: READ J: POKE I,J: NEXT I
244 DATA  104,168,104,166,223,154,72,152,72,96
252 REM   *** INITIALIZE HARDWARE ***
254 POKE AD,49: POKE AD,50
257 POKE AD + 3,GP(G1) + SP(1): POKE AD,17
260 POKE AD + 3,GP(G2) + SP(2): POKE AD,18
261 POKE AD,1
264 TEXT : HOME : GOSUB 42000: REM          RECALL "SYSCON" FILE
275 GOSUB 25000: REM   CHECK LAST SYSTEM STATUS
280 ONERR GOTO 48000
290 GOTO 10000: REM   SKIP TO MAIN MENU
900 REM   *** DRAW DATA ON PLOTTER ***
905 REM   PLOTTER ROUTINE ELIMINATED
940 RETURN

```



```

1000 REM *** PRINT STATUS DISPLAY ***
1005 TEXT : POKE 34,0: HOME
1020 VTAB 1: HTAB (40 - LEN (F$)) / 2 + 1: PRINT F$
1030 GOSUB 3000: REM PRINT EXCITATION WAVELENGTH WINDOW
1040 GOSUB 3100: REM PRINT FLUORESCENCE WAVELENGTH WINDOW
1050 GOSUB 3200: REM PRINT INTENSITY WINDOW
1060 GOSUB 3300: REM PRINT EXCITATION RANGE WINDOW
1070 GOSUB 3400: REM PRINT FLUORESCENCE RANGE WINDOW
1080 GOSUB 3500: REM PRINT READ TIME WINDOW
1090 GOSUB 3600: REM PRINT EXC. INCREMENT WINDOW
1100 GOSUB 3700: REM PRINT FLUOR. INCREMENT WINDOW
1110 GOSUB 3800: REM PRINT SCAN LENGTH WINDOW
1120 GOSUB 3900: REM PRINT TYPE SCAN WINDOW
1130 GOSUB 4000: REM PRINT OFFSET WINDOW
1140 GOSUB 4100: REM PRINT MODE WINDOW
1145 VTAB 15
1150 PRINT SK$
1155 POKE 34,15
1160 RETURN
2000 REM *** HANDLER FOR INTENSITY DISPLAY ***
2001 A$ = ""
2004 IF BA > 0 THEN GOSUB 47700: RETURN
2005 ON NB GOSUB 8000,9000: REM SINGLE & DOUBLE BEAM AUTORANGE
2015 GOSUB 3225: REM UPDATE INTENSITY DISPLAY
2050 IF KY > 127 THEN RETURN
2060 GOTO 2000
3000 REM *** EXCITATION WAVELENGTH DISPLAY ***
3010 VTAB 3: INVERSE : PRINT " EXCITATION "
3020 VTAB 4: PRINT " WAVELENGTH ": NORMAL
3030 VTAB 5: HTAB 4: PRINT EW;" "
3040 RETURN
3100 REM *** FLUORESCENCE WAVELENGTH DISPLAY ***
3110 VTAB 3: HTAB 15: INVERSE : PRINT " EMISSION "
3120 VTAB 4: HTAB 15: PRINT " WAVELENGTH ": NORMAL
3130 VTAB 5: HTAB 18: PRINT FW;" "
3140 RETURN
3200 REM *** INTENSITY DISPLAY ***
3210 VTAB 3: HTAB 29: INVERSE : PRINT " RELATIVE ";
3220 VTAB 4: HTAB 29: PRINT " INTENSITY ";; NORMAL
3225 VTAB 5: HTAB 32: IF IN < .01 THEN IN$ = STR$ (IN): PRINT VAL ( LEFT$ (IN$,2));: PRINT
RIGHT$ (IN$,4);" " : GOTO 3240
3230 VTAB 5: HTAB 32: IN$ = STR$ (IN): PRINT VAL ( LEFT$ (IN$,6));" "
3240 RETURN
3300 REM *** EXCITATION RANGE DISPLAY ***
3310 VTAB 7: INVERSE : PRINT " SCAN RANGE ": NORMAL
3330 VTAB 8: HTAB 1: R1$ = STR$ (E1) + "-" + STR$ (E2)
3332 PD = (12 - LEN (R1$)) / 2 + 1
3333 PRINT BL$;
3334 HTAB 1 + PD: PRINT R1$
3340 RETURN
3400 REM *** FLUORESCENCE RANGE DISPLAY ***

```

```

3410 VTAB 7: HTAB 15: INVERSE : PRINT " SCAN RANGE ": NORMAL
3430 VTAB 8:R2$ = STR$ (F1) + "-" + STR$ (F2)
3432 PD = (12 - LEN (R2$)) / 2 + 1
3433 HTAB 15: PRINT BL$;
3434 HTAB 15 + PD: PRINT R2$
3440 RETURN
3500 REM *** READ TIME DISPLAY ***
3510 VTAB 7: HTAB 29: INVERSE : PRINT " READ TIME ";: NORMAL
3530 VTAB 8:RT$ = STR$ (RT)
3532 PD = (12 - LEN (RT$)) / 2
3533 HTAB 29: PRINT BL$;: VTAB 8
3534 HTAB 29 + PD: PRINT RT$
3540 RETURN
3600 REM *** EXCITATION INCREMENT DISPLAY ***
3610 VTAB 10: INVERSE : PRINT " INCREMENT ": NORMAL
3630 VTAB 11:EI$ = STR$ (EI)
3632 PD = (12 - LEN (EI$)) / 2
3633 HTAB 1: PRINT BL$;
3634 HTAB 1 + PD: PRINT EI$
3640 RETURN
3700 REM *** FLUORESCENCE INCREMENT DISPLAY ***
3710 VTAB 10: HTAB 15: INVERSE : PRINT " INCREMENT ": NORMAL
3730 VTAB 11:FI$ = STR$ (FI)
3732 PD = (12 - LEN (FI$)) / 2
3733 HTAB 15: PRINT BL$;
3734 HTAB 15 + PD: PRINT FI$
3740 RETURN
3800 REM *** PRINT LENGTH OF SCAN ***
3810 VTAB 10: HTAB 29: INVERSE : PRINT "SCAN LENGTH ";: NORMAL
3830 VTAB 11:S1$ = STR$ (LS) + " (by " + STR$ (IS) + ")"
3831 PD = (12 - LEN (S1$)) / 2
3832 HTAB 29: PRINT BL$;
3835 VTAB 11: HTAB 29 + PD: PRINT S1$
3840 RETURN
3900 REM *** SCAN TYPE DISPLAY ***
3910 VTAB 13: INVERSE : PRINT "TYPE OF SCAN": NORMAL
3930 VTAB 14: HTAB 1: PRINT SC$
3940 RETURN
4000 REM *** OFFSET DISPLAY ***
4010 VTAB 13: HTAB 15: INVERSE : PRINT " OFFSET ": NORMAL
4030 VTAB 14:OF$ = STR$ (OF)
4032 PD = (12 - LEN (OF$)) / 2
4033 HTAB 15: PRINT BL$;
4034 HTAB 15 + PD: PRINT OF$
4040 RETURN
4100 REM *** MODE DISPLAY ***
4110 VTAB 13: HTAB 29: INVERSE : PRINT " MODE ";: NORMAL
4130 VTAB 14: HTAB 29: PRINT MO$
4140 RETURN
5000 REM *** VARIETY OF PROMPTS ***
5010 GOSUB 47700: PRINT "<";A$;">": RETURN

```

```

5020 PRINT : PRINT ST$ + " To accept <DEFAULTS> ---> Press "; INVERSE : PRINT "RETURN":
NORMAL : PRINT ST$: PRINT : RETURN
5030 VTAB 24: CALL - 958: PRINT "   Press "; INVERSE : PRINT "SPACEBAR"; NORMAL : PRINT " to
continue....."; GET A$: PRINT A$
5040 IF A$ < > CHR$ (32) THEN 5030
5050 HOME : RETURN
5060 VTAB 23: CALL - 958: HTAB 10: PRINT "Enter selection..."; GOSUB 47700: PRINT A$: HOME :
RETURN
5090 VTAB 23: CALL - 958: PRINT "   Press "; INVERSE : PRINT "ESC"; NORMAL : PRINT " to return
to menu..."; GET A$: PRINT A$
5100 IF A$ < > CHR$ (27) THEN 5090
5110 RETURN
5200 PRINT "   Press "; INVERSE : PRINT "ESC"; NORMAL : PRINT " to return to main menu."; RETURN
6000 REM   *** STEP MONOCHROMATOR UP ***
6010 EC = EC + 1
6020 POKE SK,2: POKE SK,0
6030 RETURN
6500 REM ** STEP MONOCHROMATOR #2 UP ***
6510 FC = FC + 1
6520 POKE SK,8: POKE SK,0
6530 RETURN
7000 REM   *** STEP MONOCHROMATOR DOWN ***
7010 EC = EC - 1
7020 POKE SK,1: POKE SK,0
7030 RETURN
7500 REM   *** STEP MONOCHROMATOR #2 DOWN ***
7510 FC = FC - 1
7520 POKE SK,4: POKE SK,0
7530 RETURN
8000 REM   **** READ ADC & AUTORANGE FOR SINGLE BEAM ****
8002 POKE - 16368,0:A$ = ""
8005 REM
8020 H1 = 0:L1 = H1
8021 FOR K = 1 TO NR
8022 KY = PEEK ( - 16384)
8024 IF KY > 127 THEN A = KY - 128:A$ = CHR$ (A): POKE - 16368,0: RETURN
8025 POKE AD,81: POKE AD,1:H1 = H1 + PEEK (H1):L1 = L1 + PEEK (LO)
8026 NEXT K
8027 N1 = (L1 + TF * H1) / NR
8032 KY = PEEK ( - 16384)
8034 IF KY > 127 THEN A = KY - 128:A$ = CHR$ (A): POKE - 16368,0: RETURN
8040 IF N1 > 4000 AND G1 < > 0 THEN G1 = G1 - 1: POKE AD + 3,GP(G1) + SP(1): POKE AD,17:
FOR J = 1 TO TD: NEXT J: GOTO 8005
8050 IF N1 < 200 AND G1 < > GM(1) THEN G1 = G1 + 1: POKE AD + 3,GP(G1) + SP(1): POKE
AD,17: FOR J = 1 TO TD: NEXT J: GOTO 8005
8052 KY = PEEK ( - 16384)
8054 IF KY > 127 THEN A = KY - 128:A$ = CHR$ (A): POKE - 16368,0: RETURN
8060 M1 = MF(G1):P1 = M1 * .00001 * INT (10000 * N1 / 410)
8065 P1 = P1 - DC(1):IN = P1
8067 DA = P1
8070 RETURN

```

```

9000 REM      *** READ ADC & AUTORANGE FOR DOUBLEBEAM ***
9005 POKE - 16368,0:A$ = ""
9020 FL = 0
9030 H1 = 0:H2 = H1:L1 = 0:L2 = L1
9050 FOR K = 1 TO NR
9052 KY = PEEK ( - 16384)
9056 IF KY > 127 THEN A = KY - 128:A$ = CHR$ (A): POKE - 16368,0: RETURN
9060 POKE AD,81: POKE AD,1:H1 = H1 + PEEK (H1):L1 = L1 + PEEK (LO)
9070 POKE AD,82: POKE AD,2:H2 = H2 + PEEK (H1):L2 = L2 + PEEK (LO)
9080 NEXT K
9090 N1 = (L1 + TF * H1) / NR
9100 N2 = (L2 + TF * H2) / NR
9120 IF N1 < 200 AND G1 < > GM(1) THEN G1 = G1 + 1: POKE AD + 3,GP(G1) + SP(1): POKE
AD,17:FL = 1
9130 IF N2 < 200 AND G2 < > GM(2) THEN G2 = G2 + 1: POKE AD + 3,GP(G2) + SP(2): POKE
AD,18:FL = 1
9140 IF FL THEN FOR J = 1 TO TD: NEXT J: GOTO 9020
9150 IF N1 > 4000 AND G1 < > 0 THEN G1 = G1 - 1: POKE AD + 3,GP(G1) + SP(1): POKE AD,17:FL
= 1
9160 IF N2 > 4000 AND G2 < > 0 THEN G2 = G2 - 1: POKE AD + 3,GP(G2) + SP(2): POKE AD,18:FL
= 1
9170 IF FL THEN FOR J = 1 TO TD: NEXT J: GOTO 9020
9180 M1 = MF(G1):M2 = MF(G2)
9181 KY = PEEK ( - 16368)
9183 IF KY > 127 THEN A = KY - 128:A$ = CHR$ (A): POKE - 16384,0: RETURN
9190 P1 = M1 * .00001 * INT (10000 * N1 / 410):P2 = M2 * .00001 * INT (10000 * N2 / 410)
9191 IF P2 = 0 THEN IN$ = " ? ":DA$ = IN$: RETURN
9192 P1 = P1 - DC(1):P2 = P2 - DC(2):IN = P1 / P2
9195 DA = .000001 * INT (1000000 * P1 / P2):DA$ = STR$ (DA)
9200 RETURN
9500 REM      **** AUTOZERO ROUTINE ****
9505 POKE AD,49
9506 SP(1) = 0:SP(2) = 0:DC(1) = 0:DC(2) = 0
9507 HOME : PRINT : HTAB 4: PRINT "Block beam and press "; INVERSE : PRINT "SPACEBAR";:
NORMAL : PRINT "...";
9509 GET A$: PRINT A$
9510 HOME : PRINT : HTAB 4: PRINT "Now checking Radiometer #1..."
9514 ON NB GOSUB 8000,9000
9516 GOSUB 9800: REM      FIND MIN NOISE
9520 IF G1 < 4 THEN INC = .244:SP(1) = 0
9522 IF G1 = 4 THEN INC = .0244:SP(1) = 16
9524 IF G1 > 4 THEN INC = .00244:SP(1) = 24
9584 PT = P1
9585 PT = PT / INC:TE = INT (PT): IF G1 = 6 AND TE > 409 THEN TE = 409
9586 AH = INT (TE / TF):AL = TE - (AH * TF): POKE AD + 1,AL: POKE AD + 2,AH: POKE AD,97: POKE
AD,33
9588 GM(1) = G1 + 1: IF GM(1) > 6 THEN GM(1) = 6:G1 = GM(1)
9590 POKE AD + 3,GP(G1) + SP(1): POKE AD,17: REM      NOW SET RAD#1 TO NEXT HIGHER RANGE
9605 L1 = 0:H1 = 0
9610 FOR K = 1 TO 500
9615 POKE AD,81: POKE AD,1:H1 = H1 + PEEK (H1):L1 = L1 + PEEK (LO)

```

```

9620 NEXT K
9625 N1 = (L1 + TF * H1) / 500
9628 M1 = MF(G1):P1 = M1 * .00001 * INT (10000 * N1 / 410)
9629 DC(1) = P1: REM SET DARK CURRENT
9630 IF NB = 1 THEN 9750
9631 HOME : PRINT : HTAB 4: PRINT "Now checking Radiometer #2..."
9632 IF G2 < 4 THEN INC = .244:SP(2) = 0
9634 IF G2 = 4 THEN INC = .0244:SP(2) = 16
9636 IF G2 > 4 THEN INC = .00244:SP(2) = 24
9644 PT = P2
9646 PT = PT / INC:TE = INT (PT): IF G2 = 6 AND TE > 409 THEN TE = 409
9648 AH = INT (TE / TF):AL = TE - (AH * TF): POKE AD + 1,AL: POKE AD + 2,AH: POKE AD,98: POKE
AD,34
9685 G2 = G2 + 1: IF G2 > 6 THEN G2 = 6
9686 GM(2) = G2
9690 POKE AD + 3,GP(G2) + SP(2): POKE AD,18: REM NOW SET RADIOMETER #2
9730 L2 = 0:H2 = 0
9734 FOR K = 1 TO 500
9736 POKE AD,82: POKE AD,2:H2 = H2 + PEEK (HI):L2 = L2 + PEEK (LO)
9738 NEXT K
9740 N2 = (L2 + TF * H2) / 500
9742 M2 = MF(G2):P2 = M2 * .00001 * INT (10000 * N2 / 410)
9745 DC(2) = P2: REM SET DARK CURRENT
9750 HOME : PRINT : PRINT " Open beam path and press "; INVERSE : PRINT "SPACEBAR";: NORMAL
: PRINT "...": GET A$: PRINT A$
9760 GOTO 10030: REM RETURN TO MAIN MENU
9800 REM ***** FIND MIN NOISE LEVEL FOR RADIOMETER #1 *****
9830 NL = 4095
9840 FOR K = 1 TO 50
9850 POKE AD,81: POKE AD,1:H1 = PEEK (HI):L1 = PEEK (LO)
9852 N1 = (L1 + TF * H1): IF N1 < NL THEN NL = N1
9855 NEXT K
9895 M1 = MF(G1):P1 = M1 * .00001 * INT (10000 * NL / 410)
9897 RETURN
9900 REM ***** FIND MIN NOISE LEVEL FOR RADIOMETER #2 *****
9930 NL = 4095
9940 FOR K = 1 TO 50
9950 POKE AD,82: POKE AD,2:H2 = PEEK (HI):L2 = PEEK (LO)
9952 N2 = (L2 + TF * H2): IF N2 < NL THEN NL = N2
9955 NEXT K
9995 M2 = MF(G2):P2 = M2 * .00001 * INT (10000 * NL / 410)
9997 RETURN
10000 REM *** MAIN MENU ***
10005 ONERR GOTO 48000
10020 GOSUB 1000: REM PRINT STATUS DISPLAY
10030 HOME : VTAB 16: HTAB 16: PRINT "MAIN MENU": PRINT
10035 HOME : REM LDX #$FB, TXS -- RESET STACK
10040 PRINT " <Z>ero set <G>o to ... NM"
10050 PRINT " <B>egin scan <F>ile management"
10060 PRINT " <C>hange status <E>xtended function"
10070 PRINT " <R>un file <Q>uit program"

```

```

10100 GOSUB 2000: REM READ KEYBOARD
10110 FOR I = 1 TO 10
10120 IF A$ = MID$ ("ZBCGPFQMRE",I,1) THEN ON I GOTO
9500,14500,11000,20500,38500,12000,38000,29000,45000,46000
10130 NEXT I
10140 GOTO 10100
11000 REM *** CHANGE STATUS MENU ***
11010 HOME : VTAB 16: HTAB 11: PRINT "CHANGE STATUS MENU": PRINT
11012 PRINT " <W>avelength <R>ead time"
11014 PRINT " <S>can range <L>ength of scan"
11016 PRINT " <I>ncrement <M>ode"
11018 PRINT " <T>ype of scan <O>ffset"
11030 PRINT
11045 GOSUB 5200: REM "ESC" KEY PROMPT
11050 GOSUB 2000: REM READ KEYBOARD
11060 FOR I = 1 TO 9
11070 IF A$ = MID$ ("SIRMTLWO" + CHR$ (27),I,1) THEN ON I GOTO
19000,19100,19200,19300,19400,19500,19600,19650,10030
11080 NEXT I
11090 GOTO 11050
12000 REM *** FILE MANAGEMENT ***
12010 HOME : VTAB 16: HTAB 12: PRINT "FILE MANAGEMENT": PRINT
12020 PRINT " <C>atalog disk <P>rint data file"
12030 PRINT " <D>elete file <R>ename file"
12040 PRINT " <L>ock file <U>nlock file"
12050 PRINT " <H>ardcopy": PRINT
12060 GOSUB 5200: REM "ESC" KEY PROMPT
12070 GOSUB 2000: REM READ KEYBOARD
12080 FOR I = 1 TO 9
12090 IF A$ = MID$ ("CDLHPRUI" + CHR$ (27),I,1) THEN ON I GOTO
24000,19700,19800,19900,26000,20000,20100,10030,10030
12100 NEXT I
12110 GOTO 12070
13500 REM TURN PRINTER ON/OFF
13510 LET PL = NOT PL
13520 GOSUB 20600: REM PRINT ON/OFF
13530 GOTO 46070: REM RETURN TO EXTENDED MENU
14500 REM *** ROUTE TO PROPER SCAN ROUTINE ***
14530 IF SC$ = "SYNCHRONOUS " THEN 17000
14545 IF SC$ = " TIME " THEN 16500
14550 GOTO 10000: REM RETURN IF NO SCAN TYPE
16500 REM *** TIME SCAN ***
16505 LET A$ = ""
16510 HOME
16511 IF D = 2 THEN PRINT " Make sure a data disk is in drive #2.": PRINT SK$
16512 PRINT : PRINT "Spectrum to be stored under what name ?"
16514 PRINT : IF A$ = "" THEN GOSUB 47500: GOTO 16516
16515 INPUT "--->";F$
16516 IF F$ = "" OR F$ = "^" THEN GOTO 10030
16517 ONERR GOTO 16524
16518 LET FT$ = F$: PRINT : PRINT D$;"VERIFY ";FT$;"D";D

```

```

16520 GOSUB 22000: REM    WARN IF FILE ALREADY EXISTS
16522 IF A$ = "C" THEN GOTO 16510
16524 PRINT PEEK (222), PEEK (219) * 256 + PEEK (218)
16691 ON NB GOSUB 8000,9000: REM  SINGLE AND DOUBLE BEAM AUTORANGE
16693 W1 = 0:W2 = LS
16700 NP = LS / IS
16705 NP = INT (NP + 1)
16710 PRINT D$;"OPEN ";F$;"D";D
16720 PRINT D$;"WRITE ";F$
16740 PRINT 2 * NP
16750 PRINT D$
16790 GOSUB 36000: REM  READ THUNDERCLOCK
16795 GOSUB 21100: REM  CONFIGURATION CHECK
16797 GOSUB 21200: REM  ESC SCAN PROMPT
16800 HOME
16810 LET NP = 1
16820 GOSUB 36200: REM  READ THUNDERCLOCK
16825 LET S0 = S2: LET S5 = 0: LET S4 = S3: LET S1 = 0
16830 ON NB GOSUB 8000,9000: REM  SINGLE & DOUBLE BEAM AUTORANGE
16835 PRINT D$;"WRITE ";F$
16840 PRINT S1: PRINT DA
16845 PRINT D$
16850 LET S7 = S1 + IS
16860 GOSUB 3230: REM  UPDATE INTENSITY DISPLAY
16870 IF S1 = 0 THEN LET MI = DA: LET MX = DA
16880 IF DA > MX THEN LET MX = DA
16890 IF DA < MI THEN LET MI = DA
16900 IF S1 > = LS THEN 16930
16910 GOSUB 36200: REM  READ THUNDERCLOCK
16915 IF S3 < > S4 THEN LET S5 = S5 + 60: LET S4 = S3
16920 LET S1 = S2 + S5 - S0
16923 IF S1 > = S7 THEN GOTO 16830
16925 GOTO 16910
16930 PRINT D$;"CLOSE ";F$
16945 HOME : PRINT : HTAB 3: PRINT "Now saving the information file..."
16950 GOSUB 30000: REM  STORE INFORMATION FILE
16952 HOME
16955 POKE 34,15
16960 GOTO 10030: REM  RETURN TO MAIN MENU
17000 REM  *** SYNCHRONOUS SCAN ***
17005 LET A$ = ""
17010 HOME
17011 IF D = 2 THEN PRINT "  Make sure a data disk is in drive #2.": PRINT SK$
17012 PRINT : PRINT "Spectrum to be stored under what name ?"
17014 PRINT : IF A$ = "" THEN GOSUB 47500: GOTO 17016
17015 INPUT "---->";F$
17016 IF F$ = "" OR F$ = "^" THEN GOTO 10030
17017 ONERR GOTO 17024
17018 LET FT$ = F$: PRINT : PRINT D$;"VERIFY ";FT$;"D";D
17020 GOSUB 22000: REM    WARN IF FILE ALREADY EXISTS
17022 IF A$ = "C" THEN GOTO 17010

```

```

17024 ONERR GOTO 48000
17026 IF FW < = 0 THEN 10030
17090 GOSUB 21100: REM  CONFIGURATION CHECK
17185 D1 = INT (E1 * 10 + .5):D2 = D1 + (OF * 10)
17187 GOSUB 21200: REM  ESC SCAN PROMPT
17190 D1 = D1 - 20:D2 = D2 - 20: GOSUB 27050:D1 = D1 + 20:D2 = D2 + 20: GOSUB 27050
17191 ON NB GOSUB 8000,9000: REM  SINGLE & DOUBLE BEAM AUTORANGE
17193 W1 = F1:W2 = F2
17200 NP = (E2 - E1) / EI
17205 NP = INT (NP + 1)
17210 PRINT D$;"OPEN ";F$;"D";D
17220 PRINT D$;"WRITE ";F$
17240 PRINT 2 * NP
17250 PRINT D$
17290 GOSUB 36000: REM  READ THUNDERCLOCK
17300 HOME
17310 NP = 1
17330 ON NB GOSUB 8000,9000: REM  SINGLE & DOUBLE BEAM AUTORANGE
17335 PRINT D$;"WRITE ";F$
17340 PRINT EW: PRINT DA
17345 PRINT D$
17350 GOSUB 3030: REM  UPDATE EXCITATION WAVELENGTH DISPLAY
17355 GOSUB 3130: REM  UPDATE EMISSION WAVELENGTH
17360 GOSUB 3230: REM  UPDATE INTENSITY DISPLAY
17370 IF NP = 1 THEN MI = DA:MX = DA
17380 IF DA > MX THEN MX = DA
17390 IF DA < MI THEN MI = DA
17400 IF EW > = E2 THEN 17430
17410 D1 = INT ((EW + EI) * 10 + .5):D2 = D1 + (OF * 10)
17415 GOSUB 27055: REM  STEP MONOCHROMATOR
17417 NP = NP + 1
17420 GOTO 17330
17430 PRINT D$;"CLOSE ";F$
17440 WD = E2
17445 HOME : PRINT : HTAB 3: PRINT "Now saving the information file..."
17450 GOSUB 30000: REM  STORE INFO FILE
17452 HOME
17455 POKE 34,15
17460 GOTO 10030: REM  RETURN TO MAIN MENU
19000 REM  *** CHANGE SCAN R^AANGE ***
19005 HOME : PRINT
19010 GOSUB 34100: REM  CHANGE SCAN RANGE
19025 GOSUB 3330: REM  UPDATE EXCITATION RANGE WINDOW
19035 GOSUB 3430: REM  UPDATE EMISSION RANGE WINDOW
19099 GOTO 10030: REM  RETURN TO MAIN MENU
19100 REM  *** CHANGE INCREMENT ***
19110 P = 6: HOME
19120 GOSUB 34200: REM  CHANGE INTERVAL
19125 GOSUB 3630: REM  UPDATE EXCITATION INCREMENT WINDOW
19130 GOSUB 3730: REM  UPDATE EMISSION INCREMENT WINDOW
19199 GOTO 10030: REM  RETURN TO MAIN MENU

```



```

19200 REM *** CHANGE READ TIME ***
19210 HOME : PRINT
19220 GOSUB 28200: REM CHANGE READ TIME
19225 GOSUB 3530: REM UPDATE READ TIME WINDOW
19299 GOTO 10030: REM RETURN TO MAIN MENU
19300 REM *** CHANGE MODE ***
19305 HOME : HTAB 13: PRINT "MODE SELECTION": PRINT
19310 HTAB 14: PRINT "<N>on-ratio"
19312 HTAB 14: PRINT "<R>atio": PRINT
19314 GOSUB 5200: REM "ESC" KEY PROMPT
19315 GOSUB 2000: REM READ KEYBOARD
19316 IF A$ = CHR$ (27) THEN 19399
19317 IF A$ = "N" THEN NB = 1
19318 IF A$ = "R" THEN NB = 2
19319 GOSUB 28192: REM SET MODE LABEL
19320 GOSUB 4130: REM UPDATE MODE WINDOW
19399 GOTO 10030: REM RETURN TO MAIN MENU
19400 REM *** SCAN TYPE MENU ***
19402 HOME : HTAB 9: PRINT "Select scan type": PRINT
19408 HTAB 5: PRINT "<S>ynchronous scan"
19411 HTAB 5: PRINT "<T>ime scan": PRINT
19412 GOSUB 5200: REM PRINT "ESC" KEY PROMPT
19414 GOSUB 2000: REM READ KEYBOARD
19415 IF A$ = CHR$ (27) THEN 19428
19420 IF A$ = "S" THEN SC$ = "SYNCHRONOUS ": GOTO 19426
19423 IF A$ = "T" THEN SC$ = " TIME ": GOTO 19426
19424 GOTO 19414
19426 GOSUB 3930: REM UPDATE SCAN TYPE DISPLAY
19428 GOTO 10030: REM RETURN TO MAIN MENU
19500 REM *** CHANGE LENGTH OF SCAN ***
19510 HOME : PRINT : PRINT "Length of scan <";LS;">": HTAB 25: GOSUB 47600
19520 IF A$ = "" THEN CALL - 998: HTAB 25: PRINT LS: GOTO 19540
19530 LS = VAL (A$)
19540 PRINT : PRINT "Interval <";IS;">": HTAB 25: GOSUB 47600
19550 IF A$ = "" THEN CALL - 998: HTAB 25: PRINT IS: GOTO 19570
19560 IS = VAL (A$)
19570 GOSUB 3830: REM UPDATE SCAN LENGTH WINDOW
19599 GOTO 10030: REM RETURN TO MAIN MENU
19600 REM *** CHANGE WAVELENGTH ***
19610 HOME : PRINT
19612 PRINT "Excitation wavelength <";EW;">": HTAB 30: GOSUB 47600
19614 IF A$ = "" THEN CALL - 998: HTAB 30: PRINT EW: GOTO 19625
19616 EW = VAL (A$):EC = INT (EW * 10 + .5)
19625 PRINT
19630 PRINT "Emission wavelength <";FW;">": HTAB 30: GOSUB 47600
19632 IF A$ = "" THEN CALL - 998: HTAB 30: PRINT FW: GOTO 19635
19634 FW = VAL (A$):FC = INT (FW * 10 + .5)
19635 GOSUB 3030: REM UPDATE EXCITATION DISPLAY
19637 GOSUB 3130: REM UPDATE EMISSION DISPLAY
19639 GOTO 10030: REM RETURN TO MAIN MENU
19650 REM *** CHANGE OFFSET ***

```

```

19652 HOME : PRINT
19654 PRINT "Amount of offset <";OF;">"; HTAB 25: GOSUB 47600
19656 IF A$ = "" THEN CALL - 998: HTAB 25: PRINT OF: GOTO 19670
19658 OF = VAL (A$)
19670 GOSUB 4030: REM  UPDATE OFFSET DISPLAY
19675 GOTO 10030: REM  RETURN TO MAIN MENU
19700 REM  *** DELETE FILE ***
19710 HOME : PRINT
19720 PRINT "Name of file to be deleted:"
19725 PRINT
19727 GOSUB 47500
19730 IF F$ = "" THEN 19799
19740 PRINT D$;"DELETE ";F$
19799 GOTO 10030: REM  RETURN TO MAIN MENU
19800 REM  *** LOCK FILE ***
19810 HOME : PRINT
19820 PRINT "Name of file to be locked:"
19825 PRINT
19830 GOSUB 47500
19840 IF F$ = "" THEN 19899
19850 PRINT D$;"LOCK ";F$
19899 GOTO 10030: REM  RETURN TO MAIN MENU
19900 REM  *** HARDCOPY OF FILE ***
19910 HOME
19920 GOSUB 23000: REM  HARDCOPY OF FILES
19999 GOTO 10000: REM  RETURN TO MAIN MENU
20000 REM  *** RENAME FILE ***
20010 HOME : PRINT
20020 PRINT "Name of file to be renamed:"
20025 PRINT
20030 GOSUB 47500
20040 IF FT$ = "" THEN 20099
20050 PRINT : PRINT "New name to give file:"
20055 PRINT
20060 GOSUB 47500
20070 IF F$ = "" THEN 20099
20080 PRINT D$;"RENAME ";FT$;" ";F$
20099 GOTO 10030: REM  RETURN TO MAIN MENU
20100 REM  *** UNLOCK FILE ***
20110 HOME : PRINT
20120 PRINT "Name of file to be unlocked:"
20125 PRINT
20130 GOSUB 47500
20140 IF F$ = "" THEN 20199
20150 PRINT D$;"UNLOCK ";F$
20199 GOTO 10030: REM  RETURN TO MAIN MENU
20500 REM  *** CALL SLEW ROUTINE ***
20510 GOSUB 27000: REM  SLEW ROUTINE
20520 GOTO 10030: REM  RETURN TO MAIN MENU
20600 REM PRINT ON/OFF FOR PRINTER
20610 VTAB 21: HTAB 22

```

```

20620 IF PL THEN INVERSE : PRINT "ON";: NORMAL : PRINT "/OFF": GOTO 20640
20630 PRINT "ON/";: INVERSE : PRINT "OFF": NORMAL
20640 RETURN
21100 REM *** CONFIGURATION CHECK ***
21110 POKE 34,0
21112 HOME : PRINT : PRINT "CONFIGURATION:": PRINT
21113 PRINT "  Light source: ";LT$: PRINT : PRINT "  Detector: ";DT$
21114 PRINT : PRINT "  Filter": PRINT "  Excitation: ";EF$: PRINT "  Emission: ";FF$: PRINT
21115 PRINT "  Slit widths": PRINT "  Excitation: ";ES$: PRINT "  Emission ";FS$: PRINT : PRINT
"  Gratings": PRINT "  Excitation: ";EG$: PRINT "  Emission: ";FG$
21116 VTAB 23: CALL - 958: HTAB 8: PRINT "Are these correct (Y/N)? ";: GOSUB 47700: IF A$ = "Y"
THEN 21122
21118 IF A$ = "N" THEN GOSUB 34300: GOTO 21122
21120 GOTO 21116
21122 HOME : GOSUB 1000: REM PRINT STATUS DISPLAY
21124 RETURN
21200 REM *** ESC SCAN PROMPT ***
21210 HOME : INVERSE : PRINT "CTRL";: NORMAL : PRINT " ";: INVERSE : PRINT "S";: NORMAL : PRINT
" to pause - ANY KEY to continue."
21212 PRINT : HTAB 8: PRINT "Press ";: INVERSE : PRINT "ESC";: NORMAL : PRINT " to cancel scan."
21214 PRINT SK$: POKE 34,19
21216 RETURN
21300 REM *** PAD SCAN TYPE WITH BLANKS ***
21310 LET TV = 12 - LEN (SC$): IF TV = 0 THEN RETURN
21320 FOR I = 1 TO TV: LET SC$ = " " + SC$: NEXT I: RETURN
22000 REM *** FILE ALREADY EXISTS ***
22010 HOME : PRINT " A file by this name already exists! ": PRINT : PRINT CHR$ (7)
22020 PRINT " You have the following options...": PRINT
22030 PRINT " <R>eplace existing data file"
22050 PRINT " <C>hange name of file for this scan"
22055 PRINT
22060 GOSUB 5200: REM "ESC" KEY PROMPT
22065 GET A$: IF A$ = CHR$ (27) THEN GOTO 10030
22070 IF A$ = "R" THEN HOME : PRINT : HTAB 3: PRINT " Old data file now being deleted.": PRINT
D$;"DELETE ";FT$: RETURN
22080 IF A$ = "C" THEN RETURN
22090 GOTO 22010
23000 REM *** HARDCOPY SELECTIONS ***
23010 HTAB 10: PRINT " HARDCOPY SELECTION ": PRINT
23040 HTAB 11: PRINT "<D>ata file"
23050 HTAB 11: PRINT "<C>atalog of disk"
23055 PRINT
23070 GOSUB 5200: REM "ESC" KEY PROMPT
23075 GOSUB 2000: REM READ KEYBOARD
23080 IF A$ = CHR$ (27) THEN 10030
23090 IF A$ = "D" OR A$ = "C" THEN GOTO 23130
23100 GOTO 23075
23130 IF A$ = "D" THEN GOSUB 23200: GOTO 23600
23140 IF A$ = "C" THEN GOSUB 23160: GOTO 23700
23160 HOME : HTAB 14: PRINT "INSTRUCTIONS": PRINT : HTAB 11: PRINT "1. Align paper": HTAB 11:
PRINT "2. Turn printer on": HTAB 11: PRINT "3. Press ";: INVERSE : PRINT "SPACEBAR": NORMAL

```

```

23170 PRINT
23180 VTAB 23: CALL - 958: GET C$: PRINT C$: IF C$ < > CHR$ (32) THEN 23180
23190 RETURN
23200 REM *** GET FILE NAME ***
23205 HOME : PRINT
23210 PRINT "Name of file to be printed:"
23220 PRINT
23230 GOSUB 47500
23235 IF FT$ = "" THEN GOSUB 1000: POP : GOTO 12010
23237 HOME
23240 RETURN
23300 REM *** TURN PRINTER ON ***
23305 HOME
23310 PRINT D$;"PR#1"
23320 PRINT CHR$ (9);"20L"
23330 PR = 1
23340 RETURN
23350 REM *** TURN PRINTER OFF ***
23360 PRINT CHR$ (9);"0L"
23370 PRINT D$;"PR#0"
23380 PR = 0
23390 RETURN
23600 REM *** HARDCOPY OF DATA FILE ***
23610 GOSUB 23160: REM PRINT INSTRUCTIONS
23620 GOSUB 23300: REM TURN PRINTER ON
23630 PRINT FT$;".": PRINT
23640 F$ = FT$: GOTO 26080
23700 REM *** HARDCOPY OF CATALOG ***
23710 PR = 1
23720 GOSUB 24000: REM PRINT CATALOG
23730 GOSUB 1000: REM PRINT STATUS DISPLAY
23740 GOTO 12010: REM RETURN TO FILE MANAGEMENT MENU
24000 REM *** CATALOG DISPLAY ***
24010 IF D = 1 THEN DD = D: HOME : GOTO 24030
24020 HOME : PRINT : HTAB 9: PRINT "Which drive? (1 OR 2) ";: GOSUB 47700: PRINT A$:DD = VAL
(A$): IF DD < 1 OR DD > 2 THEN 24020
24030 IF PR = 1 THEN PRINT D$;"PR#1": PRINT CHR$ (9);"20L"
24035 POKE 34,0: HOME
24040 HTAB 11: INVERSE : PRINT " CATALOG OF DISK ";: NORMAL : POKE 34,2
24050 IF PR = 1 THEN PRINT "DRIVE #";DD
24060 PRINT "": PRINT D$;"CATALOG,D";DD
24070 IF PR = 1 THEN PRINT CHR$ (9);"0L": PRINT D$;"PR#0":PR = 0: RETURN
24080 PRINT ST$: PRINT : PRINT
24082 VTAB 23: CALL - 958
24084 GOSUB 5200: REM ESC KEY PROMPT
24085 GET A$: PRINT A$: IF A$ < > CHR$ (27) THEN 24082
24090 POKE 34,0: HOME
24095 GOTO 10000: REM RETURN TO MAIN MENU
25000 REM *** DISPLAY LAST CONFIGURATION ***
25005 HOME : PRINT
25010 HTAB 6: INVERSE : PRINT " LAST SYSTEM CONFIGURATION ": NORMAL : PRINT

```

```

25020 PRINT ST$
25030 PRINT " Monochromator dial reading: "
25031 PRINT
25032 PRINT " Excitation....."; INVERSE : PRINT EC / 10;; NORMAL : PRINT " NM"
25034 PRINT " Emission....."; INVERSE : PRINT FC / 10;; NORMAL : PRINT " NM"
25036 ED = EC / 10:FD = FC / 10
25040 PRINT : PRINT " Grating: "
25041 PRINT
25042 PRINT " Excitation....."; INVERSE : PRINT EG$; NORMAL : PRINT " lines/mm"
25044 PRINT " Emission....."; INVERSE : PRINT FG$; NORMAL : PRINT " lines/mm"
25050 WE = .1 * (1200 / VAL (EG$)):WF = .1 * (1200 / VAL (FG$))
25055 EW = EC * WE:FW = FC * WF
25080 PRINT : PRINT " Number of disk drives: "; INVERSE : PRINT D: NORMAL
25100 PRINT : PRINT ST$
25110 VTAB 23: CALL - 958: HTAB 6: PRINT "Is this still correct? (Y/N)";
25115 GOSUB 5010: REM WAIT FOR KEYPRESS
25120 IF A$ = "Y" OR A$ = "N" THEN 25140
25130 GOTO 25110
25140 IF A$ = "N" THEN GOSUB 28000: REM INPUT NEW SYSTEM STATUS
25150 RETURN
26000 REM *** LIST DATA FILE ***
26010 HOME : PRINT
26020 PRINT "Name of data file to be listed:"
26025 PRINT
26027 GOSUB 47500
26030 IF F$ = "" OR F$ = "^" THEN 10000
26040 POKE 34,0: HOME : HTAB 20 - LEN (F$) / 2: PRINT F$: PRINT : PRINT ST$;" "; INVERSE : PRINT
"CTRL";: NORMAL : PRINT " "; INVERSE : PRINT "S";: NORMAL : PRINT " to pause - ANY KEY to continue"
26050 PRINT : PRINT " To CANCEL listing -> Press "; INVERSE : PRINT "ESC": NORMAL
26060 PRINT ST$
26080 PRINT D$;"VERIFY ";F$;"D";D
26090 PRINT D$;"OPEN ";F$;"D";D
26100 IF PR = 1 THEN PRINT "WAVELENGTH DATA VALUES": PRINT : GOTO 26120
26110 HTAB 4: INVERSE : PRINT "WAVELENGTH";: HTAB 18: PRINT " DATA VALUES ": NORMAL : PRINT
: POKE 34,10
26120 PRINT D$;"READ ";F$
26130 INPUT X:X = INT (X / 2 + .5)
26140 FOR I = 1 TO X
26150 INPUT WA: INPUT DA
26160 IF PR = 1 THEN PRINT " ";WA;" ";DA: GOTO 26180
26170 HTAB 7: PRINT WA;; HTAB 22: PRINT DA
26180 POKE - 16368,0
26190 KY = PEEK ( - 16384)
26195 IF KY = 27 THEN PRINT D$;"CLOSE": PRINT D$;"PR#0":PR = 0: GOTO 10000: REM RETURN
TO MAIN MENU
26200 NEXT I
26210 PRINT D$;"CLOSE";F$
26220 IF PR = 1 THEN PRINT CHR$ (9);"0L": PRINT D$;"PR#0":PR = 0: GOTO 10000
26240 PRINT : PRINT ST$;
26250 GOSUB 5090: REM WAIT FOR "ESC" KEYPRESS
26260 GOTO 10000: REM RETURN TO MAIN MENU

```

```

27000 REM *** GOTO WAVELENGTH ***
27010 I = PEEK ( - 16368)
27020 HOME : PRINT
27021 HTAB 13: PRINT "<1> Excitation": HTAB 13: PRINT "<2> Emission": HTAB 13: PRINT "<3> Both"
27025 PRINT : HTAB 7: PRINT "Slew which monochromator? ";: GOSUB 47700: PRINT A$:MN = VAL
(A$): IF MN < 1 OR MN > 3 THEN 27020
27030 HOME : PRINT : PRINT "Slew to what wavelength?";: GOSUB 47600: IF A$ = "" OR A$ = "^" THEN
RETURN
27040 IF MN = 1 THEN D1 = VAL (A$):D1 = INT (D1 * 10 + .5):D2 = FC: GOTO 27050
27042 IF MN = 2 THEN D2 = VAL (A$):D2 = INT (D2 * 10 + .5):D1 = EC: GOTO 27050
27045 LET J = 0: FOR I = 2 TO LEN (A$) - 1: IF MID$ (A$,I,1) = " " THEN LET J = I
27046 NEXT I: IF J = 0 THEN RETURN
27047 LET D1 = VAL ( LEFT$ (A$,J - 1)):D1 = INT (D1 * 10 + .5)
27048 LET D2 = VAL ( MID$ (A$,J + 1)):D2 = INT (D2 * 10 + .5)
27050 EW = EC * WE: GOSUB 3030: REM UPDATE EXC. WAVELENGTH DISPLAY
27052 FW = FC * WF: GOSUB 3130: REM UPDATE EM. WAVELENGTH DISPLAY
27055 IF ABS (EC - D1) < .01 AND ABS (FC - D2) < .01 THEN RETURN
27060 POKE - 16368,0
27070 IF PEEK ( - 16384) = 27 THEN PRINT D$;"CLOSE": POP : GOTO 10000
27080 IF EC < D1 THEN GOSUB 6000: REM STEP MONOCHROMATOR #1 UP
27082 IF EC > D1 THEN GOSUB 7000: REM STEP MONOCHROMATOR #1 DOWN
27090 IF FC < D2 THEN GOSUB 6500: REM STEP MONOCHROMATOR #2 UP
27092 IF FC > D2 THEN GOSUB 7500: REM STEP MONOCHROMATOR #2 DOWN
27100 GOTO 27050
28000 REM *** INPUT NEW SYSTEM STATUS ***
28010 POKE 34,0: HOME
28020 HTAB 12: INVERSE : PRINT " SYSTEM STATUS ": NORMAL
28030 GOSUB 5020: REM PRINT DEFAULT PROMPT
28035 CALL - 998
28040 PRINT "ENTER CURRENT STATUS:"
28041 GOSUB 28048: REM SET MONOCHROMATOR READING
28042 GOSUB 28110: REM SET GRATING
28045 GOSUB 28340: REM SET NUMBER OF DISK DRIVES
28046 RETURN
28048 ED = EC / 10:FD = FC / 10
28049 PRINT
28050 PRINT "Monochromator reading:"
28052 PRINT " Excitation <";ED;">";: HTAB 34
28060 INPUT A$: IF A$ < > "" THEN ED = VAL (A$)
28070 IF A$ = "" THEN HTAB 34: CALL - 998: PRINT ED
28080 IF ED < 0 THEN 28052
28090 EC = INT (10 * ED + .5)
28092 PRINT " Emission <";FD;">";: HTAB 34
28093 INPUT A$: IF A$ < > "" THEN FD = VAL (A$)
28094 IF A$ = "" THEN HTAB 34: CALL - 998: PRINT FD
28096 IF FD < 0 THEN 28092
28098 FC = INT (10 * FD + .5)
28099 WE = .1 * (1200 / VAL (EG$)):EW = EC * WE
28100 WF = .1 * (1200 / VAL (FG$)):FW = FC * WF
28105 RETURN
28110 PRINT

```

```

28120 PRINT "Grating (lines/mm):"
28122 PRINT "  Excitation <";EG$;">";: HTAB 34
28130 INPUT A$: IF A$ = "" AND EG$ = "" THEN 28122
28140 IF A$ = "" THEN HTAB 34: CALL - 998: PRINT EG$: GOTO 28170
28150 IF VAL (A$) = 0 THEN WE = 1200: GOTO 28170
28160 WE = .1 * (1200 / VAL (A$)):EG$ = A$
28170 DE = WE:EW = EC * WE
28171 PRINT "  Emission <";FG$;">";: HTAB 34
28172 INPUT A$: IF A$ = "" AND FG$ = "" THEN 28171
28173 IF A$ = "" THEN HTAB 34: CALL - 998: PRINT FG$: GOTO 28176
28174 IF VAL (A$) = 0 THEN WF = 1200: GOTO 28176
28175 WF = .1 * (1200 / VAL (A$)):FG$ = A$
28176 DF = WF:FW = FC * WF
28179 RETURN
28180 PRINT : PRINT "Number of beams to be used <";NB;">";: HTAB 34: GOSUB 47600: IF A$ = ""
THEN HTAB 34: CALL - 998: PRINT NB: GOTO 28192
28190 NB = VAL (A$): IF NB < 1 OR NB > 2 THEN 28180
28192 IF NB = 1 THEN MO$ = " NON-RATIO "
28193 IF NB = 2 THEN MO$ = "  RATIO  "
28195 GOTO 28325
28200 PRINT
28310 PRINT "Read time in seconds <";RT;">";: HTAB 34: GOSUB 47600: IF A$ = "" THEN HTAB 34:
CALL - 998: PRINT RT: RETURN
28320 LET RT = VAL (A$): IF RT < 0.014 OR RT > 40 THEN 28200
28325 LET NR = INT (150 * RT / NB)
28330 RETURN
28340 PRINT : PRINT "Number of disk drives <";D;">";: HTAB 34
28350 INPUT A$: IF A$ = "" THEN HTAB 34: CALL - 998: PRINT D: GOTO 28370
28360 D = VAL (A$)
28370 IF D < 1 OR D > 2 THEN 28340
28380 RETURN
29000 REM *** MANUAL MODE ***
29010 GOTO 10030
30000 REM *** STORE INFO FILE ***
30010 S$ = F$ + ".INFO"
30014 IF SC$ = "SYNCHRONOUS " THEN E6 = E1:E7 = E2:F6 = E1 + OF:F7 = E2 + OF
30016 IF SC$ = "  TIME  " THEN E6 = EW:E7 = EW:F6 = FW:F7 = FW
30020 IF T$ = "CLOCK BOARD NOT INSTALLED" THEN T1$ = T$:T2$ = "":T3$ = "": GOTO 30040
30030 T1$ = LEFT$ (T$, LEN (T$) - 9):T2$ = MID$ (T$, LEN (T$) - 7,2):T3$ = RIGHT$ (T$,5)
30040 PRINT D$;"OPEN ";S$;"D";D
30050 PRINT D$;"DELETE ";S$;"D";D
30070 PRINT D$;"OPEN ";S$;"D";D
30080 PRINT D$;"WRITE ";S$
30090 PRINT T1$: PRINT T2$: PRINT T3$
30092 PRINT MI: PRINT MX: PRINT NB: PRINT RT
30094 PRINT E6: PRINT E7: PRINT F6: PRINT F7
30096 PRINT EI: PRINT FI
30098 PRINT ES$: PRINT FS$
30100 PRINT EG$: PRINT FG$
30102 PRINT EF$: PRINT FF$
30130 PRINT SC$: PRINT DT$

```

```

30135 PRINT D$;"CLOSE";S$
30140 RETURN
33000 REM *** INPUT RANGE OF SCAN ***
33010 PRINT "Starting wavelength <";FW;">....."; INPUT FW$
33020 IF FW$ = "" THEN HTAB 35: CALL - 998: PRINT FW: PRINT
33030 IF FW$ = "CANCEL" OR FW$ = "^" THEN POP : GOTO 10000
33040 IF VAL (FW$) = 0 THEN RETURN
33050 FW = VAL (FW$)
33055 RETURN
33060 PRINT "Ending wavelength <";LW;">....."; INPUT LW$
33070 IF LW$ = "" THEN HTAB 35: CALL - 998: PRINT LW: HOME
33080 IF LW$ = "CANCEL" THEN POP : GOTO 10000
33090 IF LW$ = "^" THEN 33010
33100 IF VAL (LW$) = 0 THEN HOME : RETURN
33110 LW = VAL (LW$)
33120 HOME : RETURN
34100 REM *** INPUT SCAN RANGE ***
34105 HOME : HTAB 15: PRINT "SCAN RANGE": PRINT : PRINT
34110 PRINT "Excitation:"
34112 PRINT "    Beginning wavelength <";E1;">"; HTAB 35: GOSUB 47600
34114 IF A$ = "" THEN CALL - 998: HTAB 35: PRINT E1: GOTO 34120
34116 E1 = VAL (A$)
34120 PRINT "    Ending wavelength <";E2;">"; HTAB 35: GOSUB 47600
34122 IF A$ = "" THEN CALL - 998: HTAB 35: PRINT E2: GOTO 34125
34124 E2 = VAL (A$)
34125 PRINT
34130 PRINT "Emission:"
34132 PRINT "    Beginning wavelength <";F1;">"; HTAB 35: GOSUB 47600
34134 IF A$ = "" THEN CALL - 998: HTAB 35: PRINT F1: GOTO 34140
34136 F1 = VAL (A$)
34140 PRINT "    Ending wavelength <";F2;">"; HTAB 35: GOSUB 47600
34142 IF A$ = "" THEN CALL - 998: HTAB 35: PRINT F2: GOTO 34145
34144 F2 = VAL (A$)
34145 RETURN
34200 REM *** INPUT INCREMENT ***
34210 HOME : HTAB 15: PRINT "INCREMENT": PRINT : PRINT
34212 PRINT "Excitation increment <";EI;">"; HTAB 35: GOSUB 47600
34214 IF A$ = "" THEN CALL - 998: HTAB 35: PRINT EI: GOTO 34220
34216 EI = VAL (A$)
34220 PRINT
34230 PRINT "Emission increment <";FI;">"; HTAB 35: GOSUB 47600
34232 IF A$ = "" THEN CALL - 998: HTAB 35: PRINT FI: GOTO 34240
34234 FI = VAL (A$)
34240 RETURN
34300 REM *** INPUT SLIT WIDTH ***
34310 HOME : HTAB 15: PRINT "SLIT WIDTH": PRINT : PRINT
34312 PRINT "Excitation slit width <";ES$;">"
34314 PRINT : INPUT "---->";A$
34316 IF A$ = "" THEN CALL - 998: HTAB 6: PRINT ES$: GOTO 34320
34318 ES$ = A$
34320 PRINT

```



```

34330 PRINT "Emission slit width <";FS$;">"
34332 PRINT : INPUT "---->";A$
34334 IF A$ = "" THEN CALL - 998: HTAB 6: PRINT FS$: GOTO 34400
34336 FS$ = A$
34400 REM *** INPUT FILTER ***
34410 HOME : HTAB 17: PRINT "FILTER": PRINT : PRINT
34412 PRINT "Excitation filter <";EF$;">"
34414 PRINT : INPUT "---->";A$
34416 IF A$ = "" THEN CALL - 998: HTAB 6: PRINT EF$: GOTO 34420
34418 EF$ = A$
34420 PRINT
34422 PRINT "Emission filter <";FF$;">"
34424 PRINT : INPUT "---->";A$
34426 IF A$ = "" THEN CALL - 998: HTAB 6: PRINT FF$: GOTO 34500
34428 FF$ = A$
34500 REM *** INPUT GRATING ***
34510 HOME
34520 GOSUB 28110: REM GRATING INPUT
34600 REM *** INPUT LIGHT SOURCE ***
34610 HOME : PRINT
34620 PRINT "Light source <";LT$;">"
34622 PRINT : INPUT "---->";A$
34624 IF A$ = "" THEN CALL - 998: HTAB 6: PRINT LT$: GOTO 34700
34626 LT$ = A$
34700 REM *** INPUT DETECTOR ***
34710 HOME : PRINT
34720 PRINT "Detector <";DT$;">"
34722 PRINT : INPUT "---->";A$
34724 IF A$ = "" THEN CALL - 998: HTAB 6: PRINT DT$: GOTO 34730
34726 DT$ = A$
34730 RETURN
35000 REM *** PREPARE PLOTTER ***
35090 REM *** DRAW BORDERS ***
35200 REM *** LABEL X-AXIS ***
35330 REM *** PRINT GRAPH TITLE ***
35425 RETURN
36000 REM *** READ THUNDERCLOCK(TM) ***
36105 VTAB 1: HTAB 1: PRINT
36110 PRINT D$"PR#5"
36120 PRINT D$"IN#5"
36130 POKE 50,128
36140 INPUT "%";T$
36150 NORMAL
36160 PRINT D$"PR#0"
36170 PRINT D$"IN#0"
36180 RETURN
36200 REM *** READ THUNDERCLOCK (NUMERIC) ***
36210 VTAB 1: HTAB 1: PRINT
36220 PRINT D$,"PR#5"
36230 PRINT D$,"IN#5"
36240 POKE 50,128

```

```

36250 INPUT "#";S6,S6,S6,S6,S3,S2
36260 NORMAL
36270 PRINT D$;"PR#0"
36280 PRINT D$;"IN#0"
36290 RETURN
38000 REM *** ENDS PROGRAM ***
38010 HOME : HTAB 15: PRINT "QUIT MENU": PRINT
38030 HTAB 10: PRINT "<E>xit to BASIC"
38040 HTAB 10: PRINT "<B>oot another disk": PRINT
38050 GOSUB 5200: REM "ESC" KEY PROMPT
38060 GOSUB 2000: REM READ KEYBOARD
38065 IF A$ = "E" OR A$ = "B" THEN HOME : PRINT : PRINT : HTAB 11: PRINT "Now closing files...":
GOSUB 43000: REM STORE CONFIGURATION IN "SYSCON"
38070 FOR I = 1 TO 3
38080 IF A$ = MID$ ("EB" + CHR$ (27),I,1) THEN ON I GOTO 38600,38700,10030
38090 NEXT I
38100 GOTO 38060
38500 REM *** LOAD PLOT PROGRAM ***
38510 GOTO 10030
38600 REM *** RETURN TO BASIC COMMAND MODE ***
38610 HOME : PRINT " To restart program press "; INVERSE : PRINT "&";: NORMAL : PRINT " and ";:
INVERSE : PRINT "RETURN";: NORMAL : PRINT ".": END
38700 REM *** BOOT ANOTHER DISK ***
38710 HOME : PRINT : PRINT : HTAB 6: PRINT "Insert disk & press "; INVERSE : PRINT "SPACEBAR";:
NORMAL : GET A$: PRINT A$
38720 IF A$ < > CHR$ (32) THEN 38700
38730 PRINT D$;"PR#6"
39000 REM *** INTERVAL PROMPT ***
39010 PRINT "Wavelength interval must be an"
39020 PRINT " integral multiple of...";
39030 DM = (.1 * (1200 / VAL (GT$)))
39040 INVERSE : PRINT DM;" NM": NORMAL : PRINT
39050 RETURN
42000 REM *** RECALLS SYSCON FILE ***
42015 PRINT D$;"VERIFY SYSCON,D1"
42020 PRINT D$;"OPEN SYSCON,D1": PRINT D$;"READ SYSCON"
42030 INPUT D: INPUT EC: INPUT FC
42032 INPUT E1: INPUT E2: INPUT F1: INPUT F2
42036 INPUT EI: INPUT FI
42038 INPUT SC$
42039 GOSUB 21300: REM PAD SCAN TYPE WITH BLANKS
42040 INPUT OF: INPUT RT: INPUT LS: INPUT IS
42042 INPUT NB: INPUT LT$: INPUT DT$
42044 INPUT EF$: INPUT FF$
42046 INPUT ES$: INPUT FS$
42048 INPUT EG$: INPUT FG$
42049 PRINT D$;"CLOSE SYSCON"
42050 WF = .1 * (1200 / VAL (FG$)):DF = WF:WE = .1 * (1200 / VAL (EG$)):DE = WE
42053 GOSUB 28192
42055 EW = EC * WE:FW = FC * WF
42060 ED = EC / 10:FD = FC / 10

```

```

42065 LET NR = INT (150 * RT / NB)
42090 RETURN
43000 REM *** STORE SYSCON FILE ***
43020 PRINT D$;"OPEN SYSCON,D1"
43030 PRINT D$;"UNLOCK SYSCON,D1"
43040 PRINT D$;"OPEN SYSCON,D1": PRINT D$;"WRITE SYSCON"
43042 PRINT D: PRINT EC: PRINT FC
43044 PRINT E1: PRINT E2: PRINT F1: PRINT F2
43048 PRINT EI: PRINT FI
43050 PRINT SC$
43052 PRINT OF: PRINT RT: PRINT LS: PRINT IS
43054 PRINT NB: PRINT LT$: PRINT DT$
43056 PRINT EF$: PRINT FF$
43058 PRINT ES$: PRINT FS$
43060 PRINT EG$: PRINT FG$
43070 PRINT D$;"CLOSE SYSCON"
43080 PRINT D$;"LOCK SYSCON"
43100 RETURN
45000 REM RUN FILE
45010 HOME : PRINT : PRINT "Name of file?"
45020 PRINT : GOSUB 47500: IF F$ = "" OR F$ = "^" THEN 10030
45030 IF F$ = "KEYBOARD" THEN GOTO 45300
45040 IF F$ = BA$ THEN GOTO 45200
45050 PRINT D$;"OPEN ";F$;","D1": PRINT D$;"READ ";F$
45055 LET BA = 1
45060 INPUT BA$(BA)
45070 IF BA$(BA) < > "KEYBOARD" THEN LET BA = BA + 1: GOTO 45060
45080 PRINT D$: PRINT D$;"CLOSE ";F$
45090 LET BA$ = F$
45200 LET BA = 1
45210 GOTO 10030
45300 LET BA = 0
45310 GOTO 10030
46000 REM EXTENDED FUNCTION MENU
46010 HOME : VTAB 16: HTAB 10: PRINT "EXTENDED FUNCTION MENU": PRINT
46020 PRINT "      <O>pen log file"
46030 PRINT "      <C>lose log file"
46040 PRINT "      <T>en reading set"
46050 PRINT "      <P>rinter ";: GOSUB 20600
46055 PRINT
46060 GOSUB 5200: REM "ESC" KEY PROMPT
46070 GOSUB 2000: REM READ KEYBOARD
46080 FOR I = 1 TO 5
46090 IF A$ = MID$ ("OCTP" + CHR$ (27),I,1) THEN ON I GOTO 46200,46600,46700,13500,10030
46100 NEXT I
46110 GOTO 46070
46175 GOSUB 36000: REM READ THUNDERCLOCK
46200 REM OPEN LOG FILE
46205 LET A$ = ""
46210 HOME
46220 IF D = 2 THEN PRINT " Make sure a data disk is in drive #2": PRINT SK$

```

```

46230 PRINT : PRINT "Name for log file?"
46240 PRINT : IF A$ = "" THEN GOSUB 47500: GOTO 46250
46245 INPUT "---->";F$
46250 IF F$ = "" OR F$ = "^" THEN 10000
46255 ONERR GOTO 46300
46260 LET FT$ = F$: PRINT : PRINT D$;"VERIFY ";FT$;"D";D
46270 GOSUB 22000: REM WARN IF FILE ALREADY EXISTS
46280 IF A$ = "C" THEN GOTO 46210
46300 ONERR GOTO 48000
46310 LET LG$ = FT$: LET LG = 1
46320 PRINT D$;"OPEN ";LG$;"D";D
46330 GOTO 10030
46600 REM CLOSE LOG FILE
46610 LET LG$ = "": LET LG = 0: GOTO 10030
46700 REM TEN READING SET
46710 LET R(0) = 0: LET RP = 0: LET FM = 0
46715 HOME : GOSUB 36000: REM READ THUNDERCLOCK
46720 FOR I = 1 TO 10: ON NB GOSUB 8000,9000
46725 IF A = 27 THEN GOTO 10030: REM INTERRUPT FOR ESCAPE KEY
46730 LET R(I) = IN: LET R(0) = R(0) + IN
46740 IF IN < 0.01 THEN LET FM = 1
46750 NEXT I: LET R(0) = R(0) / 10
46760 FOR I = 1 TO 10: LET RP = RP + (R(0) - R(I)) ^ 2: NEXT I
46770 LET RP = SQR (RP / 9)
46775 IF FM = 1 THEN LET RP = 0: LET R(0) = 0
46780 IF LG = 1 OR PL = 1 THEN GOTO 46850
46790 HOME : PRINT "AVERAGE: ";R(0): PRINT "STD DEV: ";RP
46800 GOSUB 5200: REM ESC KEY PROMPT
46810 GET A$: PRINT A$: IF A$ < > CHR$ (27) THEN GOTO 46810
46820 POKE 34,0: HOME : GOTO 10000
46850 IF LG = 0 THEN GOTO 46900
46860 PRINT D$;"APPEND ";LG$;"D";D: PRINT D$;"WRITE ";LG$
46870 GOSUB 46950: REM PRINT INFORMATION
46880 PRINT D$: PRINT D$;"CLOSE"
46900 IF PL = 0 THEN GOTO 46940
46910 PRINT D$;"PR#1": POKE 50,128
46920 GOSUB 46950: REM PRINT INFORMATION
46930 NORMAL : PRINT D$;"PR#0"
46940 GOTO 10000
46950 REM PRINT INFORMATION
46960 PRINT CHR$ (34);T$; CHR$ (34): PRINT EW: PRINT FW
46970 PRINT R(0): PRINT RP
46980 RETURN
47500 REM INPUT "----> ";F$
47505 GOSUB 47950
47510 PRINT "---->";: LET F$ = BA$(BA)
47520 IF F$ = "USER" THEN INPUT "":F$: GOTO 47530
47525 PRINT
47530 GOTO 47900
47600 REM INPUT A$
47605 GOSUB 47950

```

```

47610 LET A$ = BA$(BA): IF A$ = "USER" THEN INPUT "";A$
47620 GOTO 47900
47700 REM GET A$
47705 GOSUB 47950
47710 LET A$ = BA$(BA): IF A$ = "USER" THEN GET A$
47720 GOTO 47900
47900 REM ADJUST BA
47910 LET BA = BA + 1: IF BA = 1 THEN LET BA = 0
47920 RETURN
47950 POKE - 16368,0: IF PEEK ( - 16384) < > 27 THEN RETURN
47960 POKE 34,0: HOME : LET BA = 0: GOTO 10000
48000 REM *** ERROR HANDLING ROUTINE ***
48010 PRINT D$;"CLOSE": LET BA = 0: PRINT D$;"PR#0"
48020 PRINT CHR$ (7): PRINT CHR$ (7)
48030 POKE 34,0: HOME : VTAB 5
48032 PRINT PEEK (222)
48034 PRINT 256 * PEEK (219) + PEEK (218)
48045 E1$ = "":E2$ = ""
48047 E = PEEK (222)
48050 IF E = 4 THEN E1$ = "The disk is write protected...":E2$ = "Remove the write protect tab."
48060 IF E = 5 THEN E1$ = "More data was expected...":E2$ = "Check the data file by listing."
48070 IF E = 6 THEN E1$ = "The file is not on the disk...":E2$ = "Check the disk catalog."
48080 IF E = 8 THEN E1$ = "A disk error has occurred...":E2$ = "Check the disk and drive."
48090 IF E = 9 THEN E1$ = "The disk is full...":E2$ = "Replace with an initialized disk."
48100 IF E = 10 THEN E1$ = "The file is locked...":E2$ = "Please unlock the file first."
48110 IF E = 42 THEN E1$ = "More data was expected...":E2$ = "Please check the data by listing."
48120 IF E = 133 THEN E1$ = "A division by zero was attempted...":E2$ = STR$ ( PEEK (219) * 256 +
PEEK (218))
48130 IF E = 255 THEN E1$ = "The program has been exited...":E2$ = ""
48132 IF E1$ = "" THEN E1$ = "An error has been encountered...":E2$ = "Refer to reference manual!"
48135 INVERSE
48140 HTAB 13: PRINT "ERROR CODE #";E: PRINT : PRINT
48142 NORMAL : PRINT ST$
48150 PRINT E1$: PRINT : PRINT E2$
48155 PRINT : PRINT ST$
48160 GOSUB 5090: REM WAIT FOR KEYPRESS
48170 GOTO 10000: REM RETURN TO MAIN MENU

```

APPENDIX E

A Model of a Solid Substrate Fermentation

E.1) Introduction and Objectives of the Modeling Studies

The modeling studies were undertaken for the following reasons:

- 1) To quantify the major mechanisms controlling protease production, specifically the effect of glucose and amino acids, and toxic product accumulation.
- 2) To design a logical operating strategy (solid substrate feeding profile) for the fermentation.
- 3) To test the operating strategy's ability to increase productivity.
- 4) To identify a hierarchy of limitations that prevent the theoretical maximum protease production capability of the microorganism, from being reached.

E.2) Literature Survey.

Solid substrates can be utilized during fermentation using one of two mechanisms. In the first mechanism, the cell is directly attached to the solid surface with contact required for degradation. This is the mechanism predominant in most solid state fermentations where the substrate is a moist solid (Tengerdy, 1985). In the second mechanism, the microorganism is uniformly distributed in the continuous aqueous phase. The cells excrete an enzyme into the fermentation broth, which after transport to the solid surface, degrades the solid substrate. In solid substrate submerged culture, adhesion of the microorganism to

the solid surface is not significant and the second mechanism predominates (Hsu, 1987). This mechanism will be considered in more detail.

The mechanism of solid substrate utilization in submerged culture can be subdivided into three sections as follows:

- 1) The production of the degradative enzyme(s).
- 2) The degradation of the solid substrate.
- 3) The utilization of solubilized components.

The factors that affect the production of degradative enzymes include:

- the types and quantities of enzymes produced by the cell as mediated by induction and repression.
- diffusion of these enzymes across the boundary layer surrounding the cell.
- convection of the enzymes in the bulk fluid.
- inhibition of enzyme activity by any inhibitors that may be present in the liquid medium.

The factors that affect the degradation of the solid substrate include:

- the susceptible surface area including particle size, porosity, composition and homogeneity of the solid.
- diffusion of the enzymes and products across the boundary layer surrounding the solid including pore diffusional resistance.
- enzyme adsorption and desorption at the solid surface, including the mobility of the enzyme at the solid surface.
- the kinetics of enzyme reaction at the solid surface.

- product inhibition and inactivation of the enzyme.
- degradation of large fragments to small monomers (in the protein example, the degradation of polypeptides to amino acids).

The factors that affect the utilization of solubilized components include:

- Diffusion of amino acids across the boundary layer surrounding the cell.
- The kinetics of uptake of the substrate and its metabolic utilization.
- External factors affecting the cell, including inhibition of growth by substrate, product or other component.
- Limitations caused by other nutrients, for example, oxygen.

The above described mechanism is very complex. Constructing a model to describe this situation is approached using two techniques. First, all the effects are evaluated and only the dominant or major effects are considered. A series of assumptions are usually listed. This allows simplification of the complex situation to a point where it can be easily handled. Secondly, poorly described or unknown variables are often lumped together to allow them to be handled by existing theory. Each of the above three listed sections of the model will be discussed with respect to assumptions used to simplify the actual situation.

The production of degradative enzymes.

Since the conditions within a fermentor are usually very turbulent, product formation is usually assumed to be solely a function of growth and/or cell concentration. The Ludeking and Piret model (Ludeking and Piret, 1959a,b) is perhaps the most widely used model to describe product formation. This model links the product formation rate to the

cell growth and cell concentration as follows:

$$\frac{dP}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X \quad (\text{E1})$$

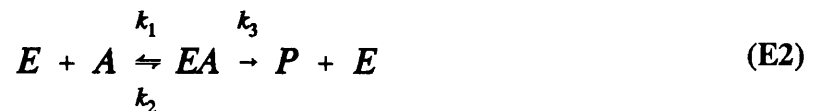
where:

- P** : product concentration
t : time
X : cell concentration
 α : growth-associated constant
 β : non-growth-associated constant

The value of α and β allow either growth-associated, non-growth-associated or mixed-growth-associated product formation to be described.

The degradation of the solid substrate

The kinetics of enzyme degradation of solid substrate has been evaluated (Bailey and Ollis, 1986; McLaren and Packer, 1970) using the following reaction pathway:



where:

- E** : free enzyme in solution
A : vacant adsorption site
EA : enzyme-substrate complex
P : product
t : time
 k_1, k_2, k_3 : reaction rate constants

It is usually assumed that adsorption/desorption is at equilibrium and that product formation is an irreversible decomposition. A mass balance is then written for the enzyme and adsorption sites as follows:

$$E_o = E + EA \quad (E3)$$

E_o : total enzyme

$$A_o = A + EA \quad (E4)$$

A_o : total adsorption sites

A steady state balance is now performed on the enzyme-substrate complex as follows:

$$\frac{dEA}{dt} = k_1 A.E - k_2 EA - k_3 EA = 0 \quad (E5)$$

which upon substituting the enzyme and site mass balances and rearranging gives:

$$k_1 (E_o - EA) (A_o - EA) - (k_2 + k_3) EA = 0 \quad (E6)$$

There are now two extreme cases which can simplify the above equation

- 1) The substrate is in excess ($A_o \gg E_o, \Rightarrow A_o = A$)
- 2) The enzyme is in excess ($E_o \gg A_o, \Rightarrow E_o = E$)

The reaction rate expression for the substrate in excess becomes:

$$V = \frac{dP}{dt} = k_3 EA = \frac{k_3 E_o A_o}{K_M + A_o} \quad (E7)$$

The reaction rate expression for the enzyme in excess becomes:

$$V = \frac{dP}{dt} = k_3 \cdot EA = \frac{k_3 \cdot E_o \cdot A_o}{K_M + E_o} \quad (\text{E8})$$

where:

$$K_M = \frac{k_2 + k_3}{k_1} \quad (\text{E9})$$

V : Reaction rate (product formation rate)

For solid substrate reactions it is common for the enzyme to be in excess, whereas for reactions in solution it is usual that the substrate is in excess (Bailey and Ollis, 1986). Note the case of the substrate being in excess is the Michaelis-Menten equation.

The degradation of solid fish protein has been studied for three reasons. The first was a group of studies in the 1970s looking at the solubilization of fish protein concentrate (Hevia et al., 1976; Archer et al., 1973; Cheftel et al., 1971). Fish protein concentrate was a protein source of nutritive value but it had very poor functional properties and so it could not be incorporated into food. Its major drawback was that it was not soluble or dispersable and had poor swelling and wetting characteristics. Thus the enzymatic degradation of fish protein concentrate was investigated to try to obtain a soluble product.

The second group of studies occurred in the mid-late 1980s and studied the proteolytic degradation of stickwater. Stickwater is a fish protein and is used in the manufacture of fishmeal. The main problem was that stickwater becomes very viscous during drying. Proteolytic degradation of stickwater lowered the viscosity and saved

considerable energy costs (Schaffeld et al., 1989; Jacobsen, 1985; Jacobsen and Lykke-Rasmussen, 1984).

A third study occurred recently and looked at the proteolytic solubilization of solid fish protein in waste streams (Venugopal et al., 1989).

Most of the above studies looked at the effect of various operating conditions on protein solubilization in order to obtain the best operating conditions for their particular process. The only detailed study of the enzyme kinetics of fish protein degradation was that of Archer et al., 1973, who noted that the overall kinetics could be described as a sequence of two first order reactions. An initial fast reaction occurred in which loosely bound polypeptide chains are cleaved from an insoluble protein particle, followed by a second, slower reaction in which the more compacted core protein is digested.

The utilization of solubilized components

The most common way to describe substrate utilization is to perform a balance around the fermentor (Wang et al., 1979).

$$\text{Substrate accumulation} = \text{Substrate feed} - \text{Growth} - \frac{\text{Product}}{\text{synthesis}} - \text{Maintenance} - \text{Substrate removal} \quad (\text{E10})$$

$$\frac{dS}{dt} = \frac{F_i S_o}{V} - \frac{\mu X}{Y_{X/S}} - \frac{q_P X}{Y_{P/S}} - mX - \frac{F_o S}{V} \quad (\text{E11})$$

where:

S : substrate concentration in fermentor

S_o : substrate concentration in feed

t : time

F_i : feed rate to fermentor

F_o : removal rate from fermentor

V : liquid volume of fermentor

q_P : specific productivity

X : cell concentration

m : maintenance coefficient

μ : growth rate

Y_{X/S} : cell yield on substrate

Y_{P/S} : product yield on substrate

In batch fermentation during rapid growth often product formation and maintenance may be small enough to be neglected. In this case, substrate utilization is related to the growth rate, cell concentration and the cell yield on substrate.

The Monod equation (Monod, 1949) is widely used to predict the growth rate. The growth rate is based on the growth limiting substrate concentration as follows.

$$\mu = \frac{\mu_{\max} \cdot S}{K_s + S} \quad (\text{E12})$$

where:

- μ : specific growth rate
- μ_{\max} : maximum specific growth rate
- S : growth limiting substrate concentration
- K_S : saturation constant

E.3) Overview and Structure of the Model.

The structure of the model can be seen in figure E1. Initially, the model was constructed to take into account only the effects of amino acids and glucose on protease production. Later the effects of the toxic products, acetate and acetoin, were included. Acetate and acetoin inhibited the growth of *Bacillus subtilis*. Because it was difficult to quantify the effects of the numerous individual amino acids produced from the proteolytic degradation of the fishmeal, all the amino acids were lumped together and call dissolved fishmeal.

There are several assumptions implicit in the model structure.

- 1) All the amino acids from the degradation of fishmeal have equal effectiveness at decreasing protease production.
- 2) The only growth inhibitory compounds are acetate and acetoin. Other end products, such as 2,3-butanediol, have a negligible growth inhibitory effect at typically encountered concentrations.
- 3) Glucose and amino acids negatively regulate both neutral and alkaline protease formation.

The only assumption that is not supported in the literature is that all amino acids are equally effective at decreasing protease production. In fact, evidence exists to the contrary. Medium with the same dissolved fishmeal concentrations may have different regulation ability because of a different amino acid profile. This assumption was made, however, because of the difficulty in measuring and analyzing the amino acid profile of the medium. Thus to make the model simple enough to be meaningful all the amino acids were lumped together as dissolved fishmeal.

The model was developed by constructing a material balance around each of the components in the system.

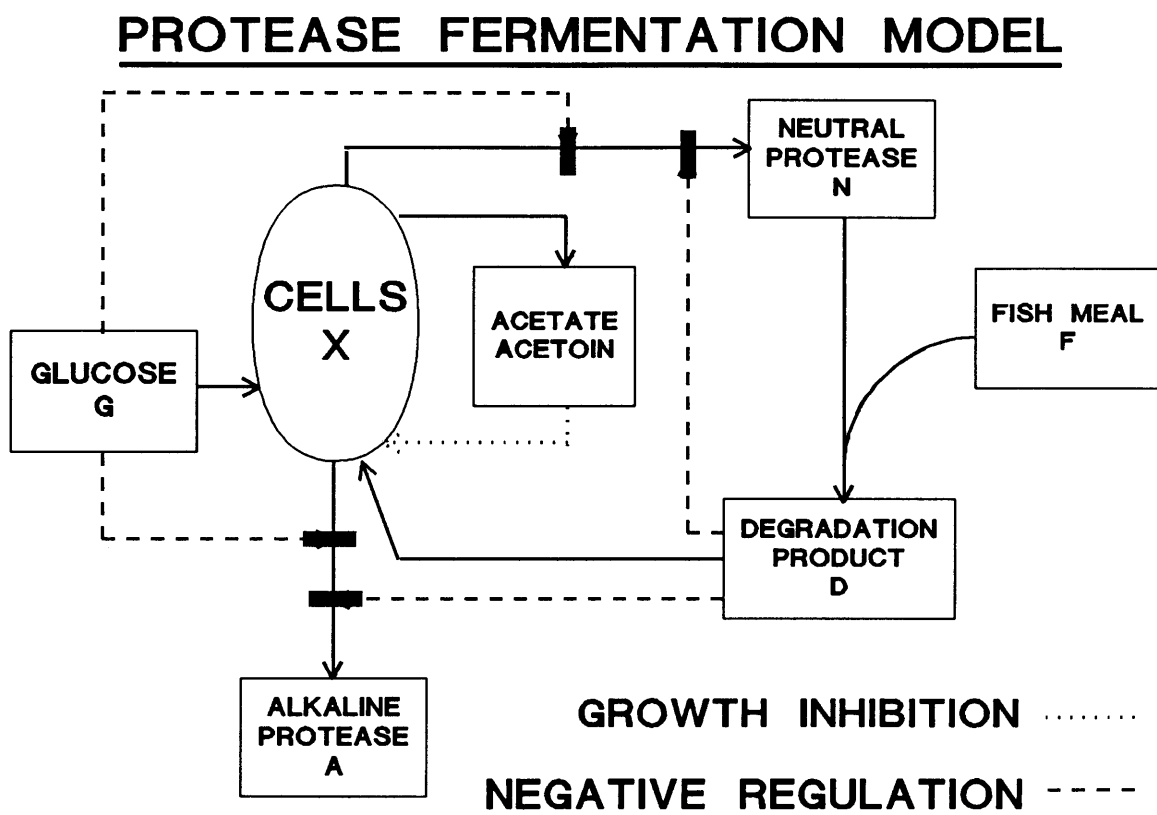


Figure E1: The structure of the solid substrate, protease fermentation model.

E.4) Balance on Dissolved Fishmeal

The source of the dissolved fishmeal is from the degradation of the solid fishmeal and the dissolved fishmeal is used by the cell for growth and product formation. This can be represented as follows, assuming that proteases are the main products:

$$\begin{array}{c} \text{DISSOLVED} \\ \text{FISHMEAL} \\ \text{ACCUMULATION} \end{array} - \begin{array}{c} \text{FROM} \\ \text{FISH} \\ \text{MEAL} \end{array} - \left[\begin{array}{c} \text{USED} \\ \text{FOR} \\ \text{GROWTH} \end{array} + \begin{array}{c} \text{USED FOR} \\ \text{NEUTRAL} \\ \text{PROTEASE} \end{array} + \begin{array}{c} \text{USED FOR} \\ \text{ALKALINE} \\ \text{PROTEASE} \end{array} \right] \quad (\text{E13})$$

$$- \frac{dD}{dt} - \frac{dF}{dt} + \left[\frac{1}{Y_{X/D}} \frac{dX}{dt} + \frac{1}{Y_{N/D}} \frac{dN}{dt} + \frac{1}{Y_{A/D}} \frac{dA}{dt} \right] \quad (\text{E14})$$

where

D : dissolved fishmeal (g/l)

F : fishmeal (g/l)

t : time (h)

X : cell concentration (g/l)

N : neutral protease (NPU)

A : alkaline protease (APU)

$Y_{X/D}$: yield coefficient (g cell/g dissolved fishmeal)

$Y_{N/D}$: yield coefficient (NPU/g dissolved fishmeal)

$Y_{A/D}$: yield coefficient (APU/g dissolved fishmeal)

The difficulty with equation E14 is estimating the coefficients $Y_{X/D}$, $Y_{N/D}$, and $Y_{A/D}$.

To overcome this problem two assumptions were made.

- 1) Most of the fishmeal is used for growth. This assumption neglects any dissolved fishmeal flux that goes into protease formation. This is a reasonable assumption

because in the batch, fed-batch and control fermentations, to which this model was applied, the weight ratio of cells to cells plus protease at the end of the fermentations were 92%, 73% and 91% respectively. The actual concentrations of protease, as opposed to their activities, was estimated by comparing their activities to those of known quantities of Dispase (Boehringer Mannheim Biochemicals, grade 2, Cat# 165-859, Lot# 10960325-29), a purified neutral protease from *Bacillus polymyxa*, and Nagarase (Sigma Chemical Company, Cat # P-4789, type 27, Lot# 97F-0218), a purified alkaline protease from *Bacillus subtilis*. This assumption reduces equation E14 to the following form.

$$-\frac{dD}{dt} = \frac{dF}{dt} + \frac{1}{Y_{\frac{X}{D}}} \cdot \frac{dX}{dt} \quad (\text{E15})$$

2) That the fermentations ended because the dissolved fishmeal has been depleted.

Although there is no way to rigorously test this assumption, it seems reasonable because the other nutrients were in excess. If we assume that the dissolved fishmeal concentration was zero at the end of the fermentation then we have a means of estimating $Y_{X/D}$. At the end of the fermentation the cell accumulation will be proportional to the fishmeal degraded since none of the fishmeal is left in the medium as dissolved fishmeal. $Y_{X/D}$ can be calculated as follows:

$$-\Delta F = \frac{1}{Y_{\frac{X}{D}}} \cdot \Delta X \quad (\text{E16})$$

$$\Rightarrow Y_{\frac{X}{D}} = \frac{\Delta X}{-\Delta F} \quad (\text{E17})$$

$Y_{X/D}$ was determined by plotting the points corresponding to the end of four fermentations and calculating an average slope, see figure E2. A typical dissolved fishmeal profile in a batch fermentation based on the determined $Y_{X/D}$ value can be seen in figure E3. The dissolved fishmeal profile predicted by the model was compared to the measured nitrogen content of the liquid phase (cell and solid free) obtained during a batch fermentation, see figure E3. The nitrogen content was measured by Galbraith Laboratories Inc., 2323 Sycamore Dr., Knoxville, TN 37921-1750. Both the nitrogen content and the predicted dissolved fishmeal concentration follow the same trend.

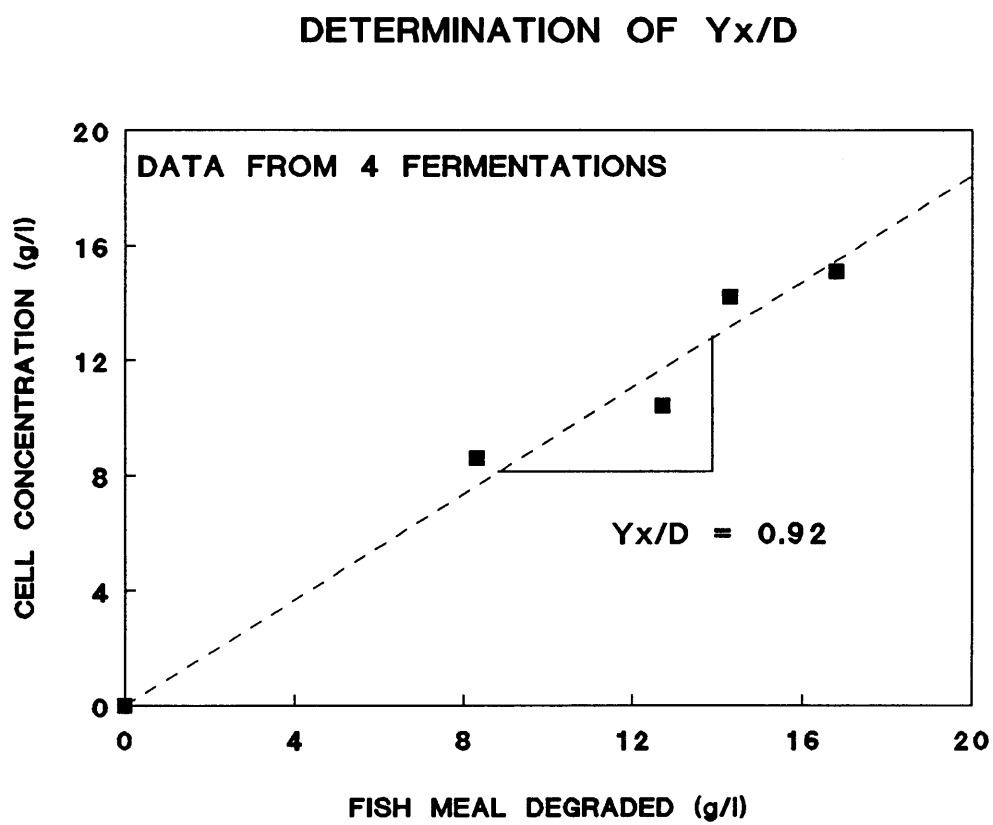


Figure E2: The calculation of $Y_{x/D}$.

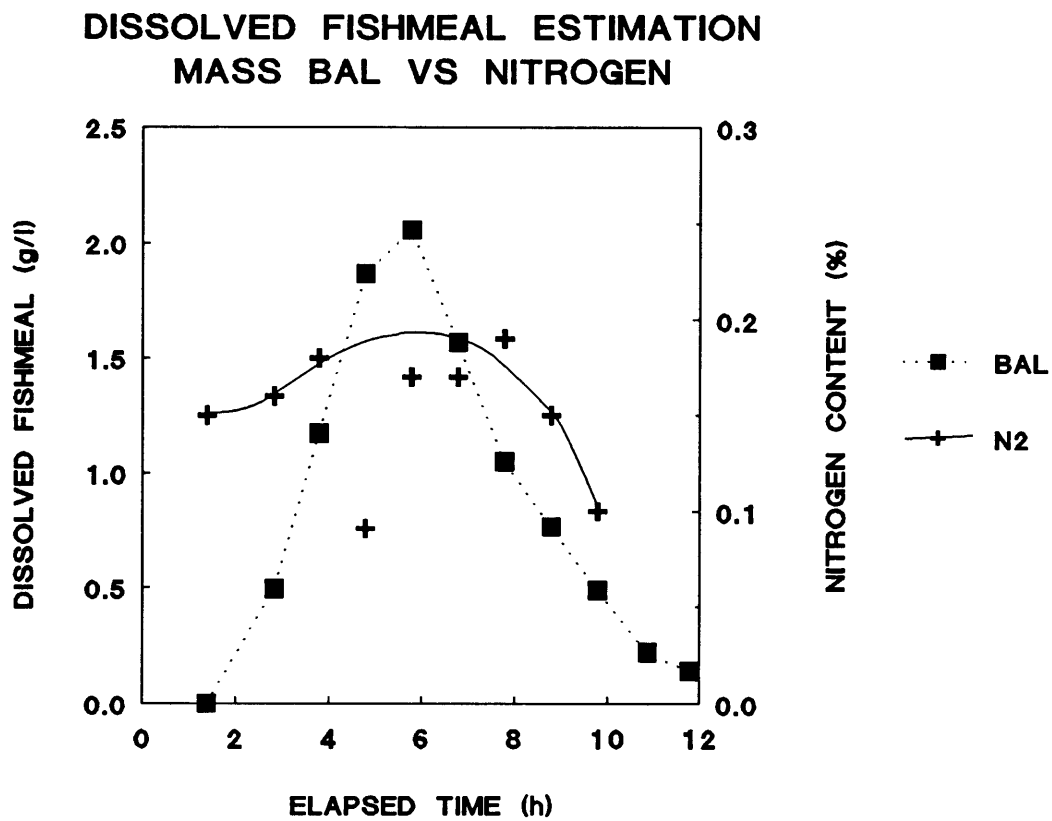


Figure E3: A typical dissolved fishmeal profile predicted for a batch fermentation.

E.5) Balance on Cells

The balance on cells assumes that the fishmeal is limiting the growth. The cell concentration is estimated using the following equation.

$$\frac{dX}{dt} = \mu \cdot X \quad (\text{E18})$$

The growth rate is assumed to be predicted by the Monod equation, see equation E19.

$$\mu = \frac{\mu_{MAX} \cdot D}{K_D + D} \quad (\text{E19})$$

where:

μ : specific growth rate

μ_{MAX} : maximum specific growth rate

K_D : constant

Substituting equation E19 into equation E18 gives

$$\frac{dX}{dt} = \mu_{MAX} \cdot \frac{D}{K_D + D} \cdot X \quad (\text{E20})$$

The cell concentration used in the model was the average of the results predicted by the light scatter and DNA assays in the batch and fed-batch fermentations and by the carbon dioxide evolution technique in the control fermentation. The carbon dioxide evolution technique was used for the control fermentation because the light scatter assay was unavailable and the DNA assay could not be used because of interference by large

amounts of fishmeal. It was assumed that glucose did not limit the growth of the cells.

The maximum specific growth rate was measured independently by assuming that the growth rate in equation E18, was constant during the initial growth of *Bacillus subtilis* on nutrient broth. The determination of μ_{MAX} during one such fermentation can be seen in figure E4. The value of μ_{MAX} was determined in three fermentations and the values averaged. The fit of the Monod equation to the data to determine K_D can be seen in figure E5. There is considerable scatter in figure E5 but the general trend can be seen. The data shown in figure E5, from solid substrate fermentations is insufficient to determine if the above predicted value of μ_{MAX} is accurate.

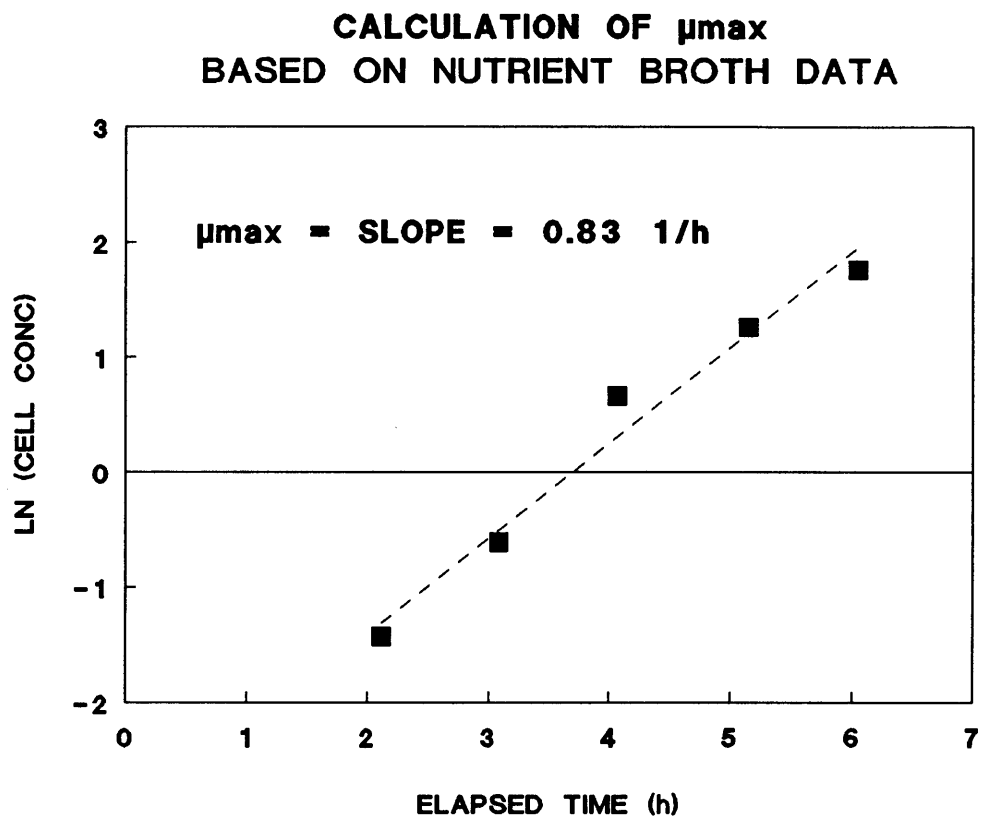


Figure E4: The determination of the maximum specific growth rate.

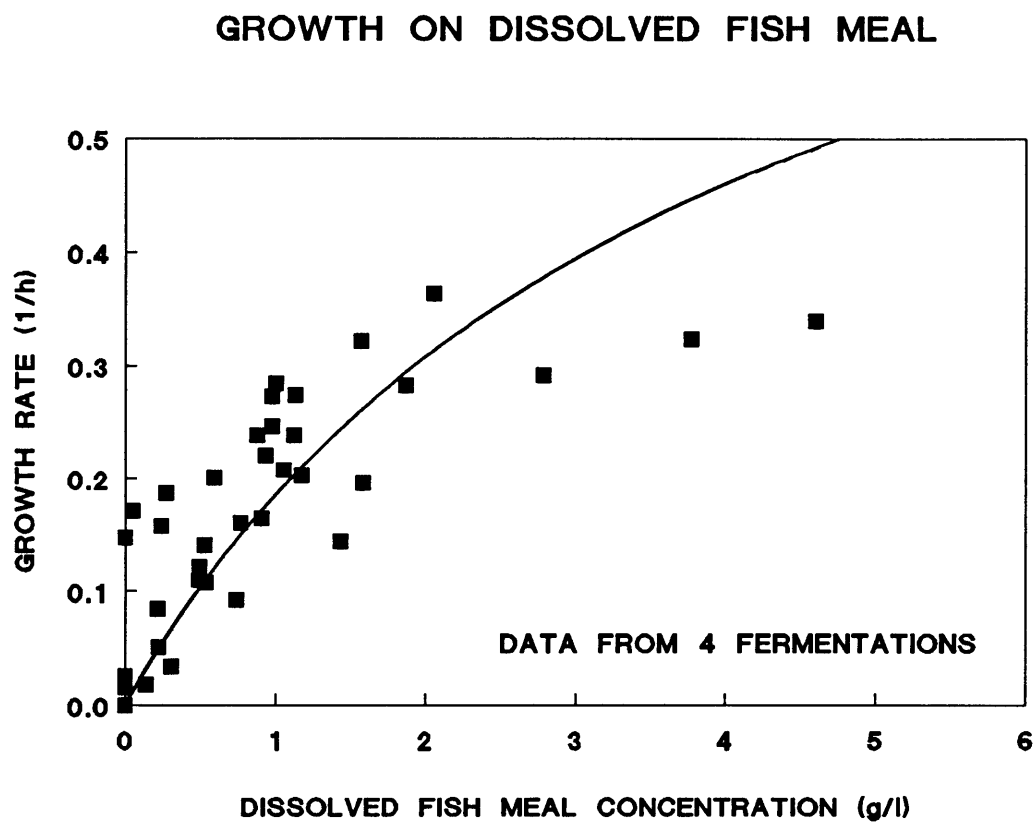


Figure E5: The fit of the solid substrate fermentation data to the Monod equation.

E.6) Balance on Glucose

A balance on glucose can be written as follows:

$$\begin{array}{c} \text{GLUCOSE} \\ \text{ACCUMULATED} \end{array} = \begin{array}{c} \text{GLUCOSE} \\ \text{ADDED} \\ \text{DURING} \\ \text{FERM} \end{array} - \begin{array}{c} \text{GLUCOSE} \\ \text{CONSUMED} \\ \text{DURING} \\ \text{FERM} \end{array} \quad (\text{E21})$$

The glucose consumption is assumed proportional to cell accumulation. This is a reasonable assumption as can be seen in figure E6 and yields the following equation:

$$(\text{E22}) \quad -\frac{dG}{dt} = -\frac{dG_A}{dt} + \frac{1}{K_{\frac{X}{G}}} \cdot \frac{dX}{dt}$$

where:

G : glucose (g/l)

G_A : glucose added (g/l)

$K_{X/G}$: constant relating glucose consumption to cell accumulation (g/g)

Upon further investigation it was observed that the constant $K_{X/G}$ may be a function of dissolved fishmeal concentration, see figure E7. This makes intuitive sense since the cell is capable of getting carbon from the dissolved fishmeal as well as from glucose. Thus the more dissolved fishmeal available to the cell the less would come from the glucose and hence cell accumulation per unit consumption of glucose would rise. Thus equation E23 was fitted to the data. However, the data shown in figure E7 is open to interpretation and some could argue that a constant $K_{X/G}$ value is a better fit.

$$K_{\frac{X}{G}} = K_{\alpha} + \frac{K_{\beta} \cdot D}{K_{\gamma} + D} \quad (\text{E23})$$

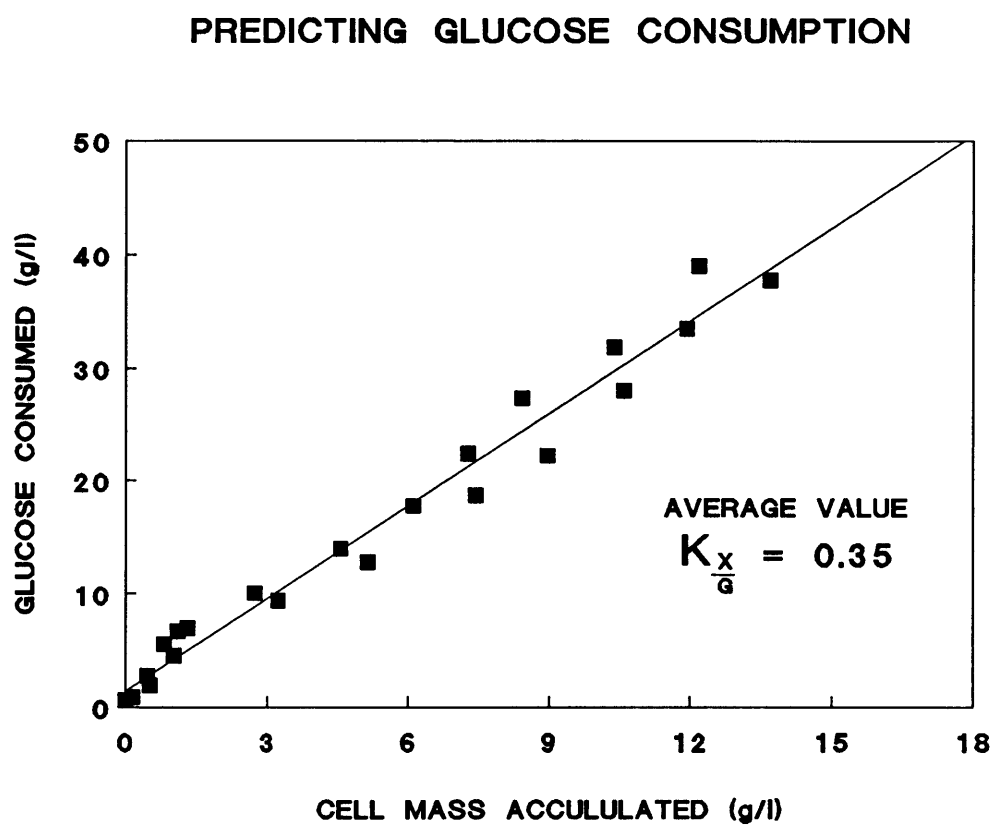


Figure E6: Calculation of glucose consumption based on cell mass accumulation.

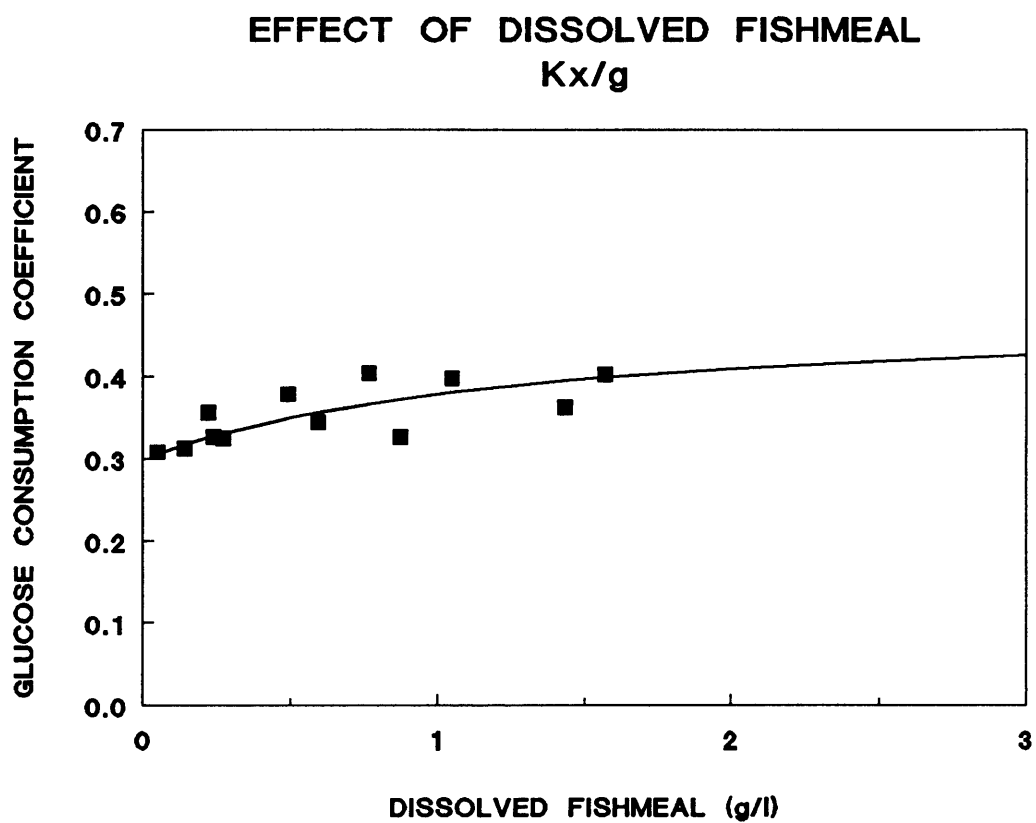


Figure E7: The influence of dissolved fishmeal concentration on $K_{x/g}$.

E.7) Balance on Fishmeal

The accumulation of fishmeal is a balance between fishmeal added and fishmeal degradation as follows:

$$\text{FISHMEAL ACCUMULATION} = \text{FISHMEAL ADDITION} - \text{FISHMEAL DEGRADATION} \quad (\text{E24})$$

$$-\frac{dF}{dt} = -\frac{dF_A}{dt} + \gamma \cdot F \quad (\text{E25})$$

where

F_A : fishmeal added (g fishmeal/l)

γ : degradation rate (g fishmeal/g fishmeal-h)

Applying equation (E8) which has been used to describe solid substrate enzymatic degradation we have:

$$\gamma = \frac{\gamma_{MAX} \cdot A_R \cdot N}{K_F + N} \quad (\text{E26})$$

where:

γ_{MAX} : maximum area specific degradation rate (g fishmeal/g fishmeal-m²-h)

A_R : surface area of particle (m²)

K_F : constant (NPU)

Estimating the area in equation E26 is difficult, especially since the fishmeal particles do not degrade as uniform spheres, see figures E8 to E11. To overcome this difficulty a general relationship relating area to fishmeal concentration was assumed as follows:

$$A_R = \psi \cdot F^{(B-1)} \quad (\text{E27})$$

where

ψ : constant ($\text{m}^2 \cdot \text{l}^{(B-1)} / \text{g fishmeal}^{(B-1)}$)

B : constant (unitless)

Substituting equations E27 and E26 into equation E25 gives:

$$-\frac{dF}{dt} = -\frac{dF_A}{dt} + \frac{\gamma_{MAX} \cdot \psi \cdot F^B \cdot N}{K_N + N} \quad (\text{E28})$$

If the neutral protease concentration is constant and $dF_{A/dt}$ is 0, then the following relationship applies :

$$-\frac{dF}{dt} \propto F^B \quad (\text{E29})$$

Equation E29 can now be solved for B, see figure E12. A B value of 1.9 was obtained which is different from the 1.67 value which would be expected if a shrinking sphere model described the solid substrate breakdown.

The coefficient K_F can now be evaluated when dF_A/dt is 0 (see figure E13), from a saturation type plot as follows:

$$-\frac{dF}{dt} \cdot \frac{1}{F^B} = \frac{\psi \cdot \gamma_{MAX} \cdot N}{K_F + N} \quad (\text{E30})$$

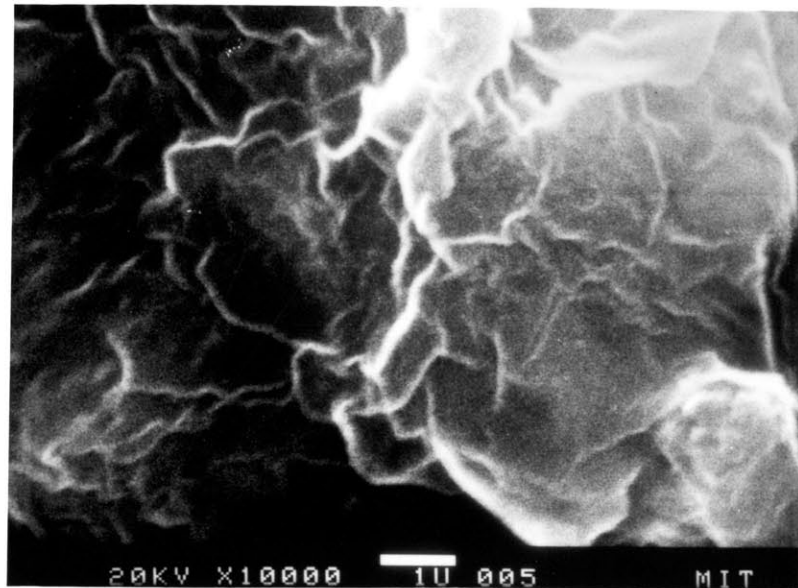


Figure E8: A SEM photograph showing fishmeal prior to fermentation.

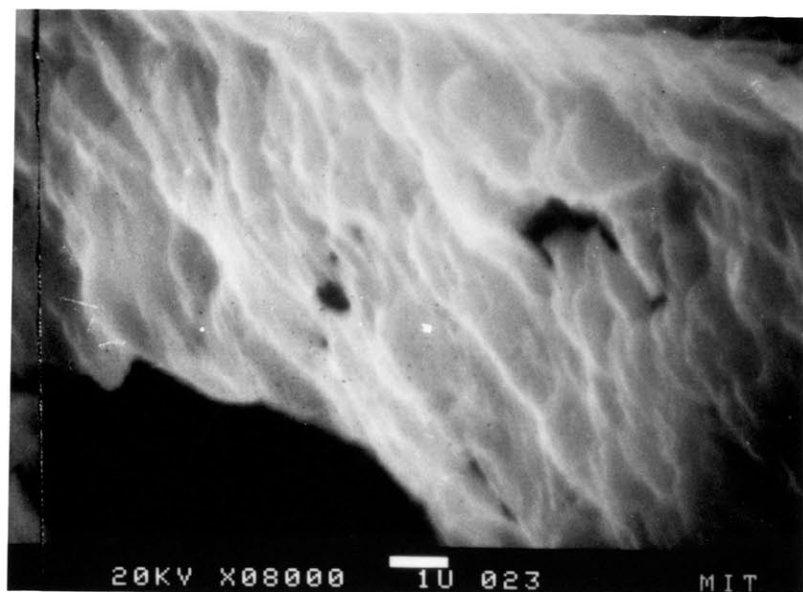


Figure E9: A SEM photograph showing the initial degradation of the fishmeal. Pits in the fishmeal are beginning to form.

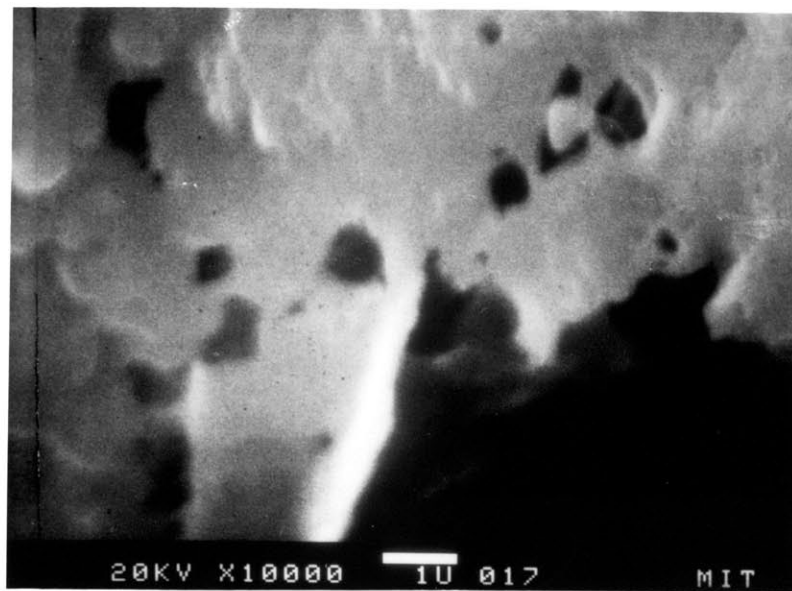


Figure E10: A SEM photograph showing more extensive pit formation.

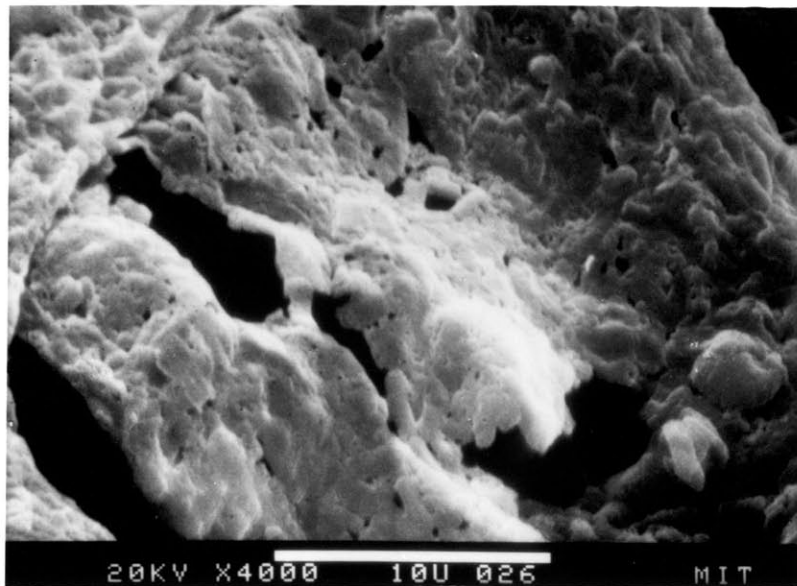


Figure E11: A SEM photograph showing that holes have formed in the fishmeal particles.

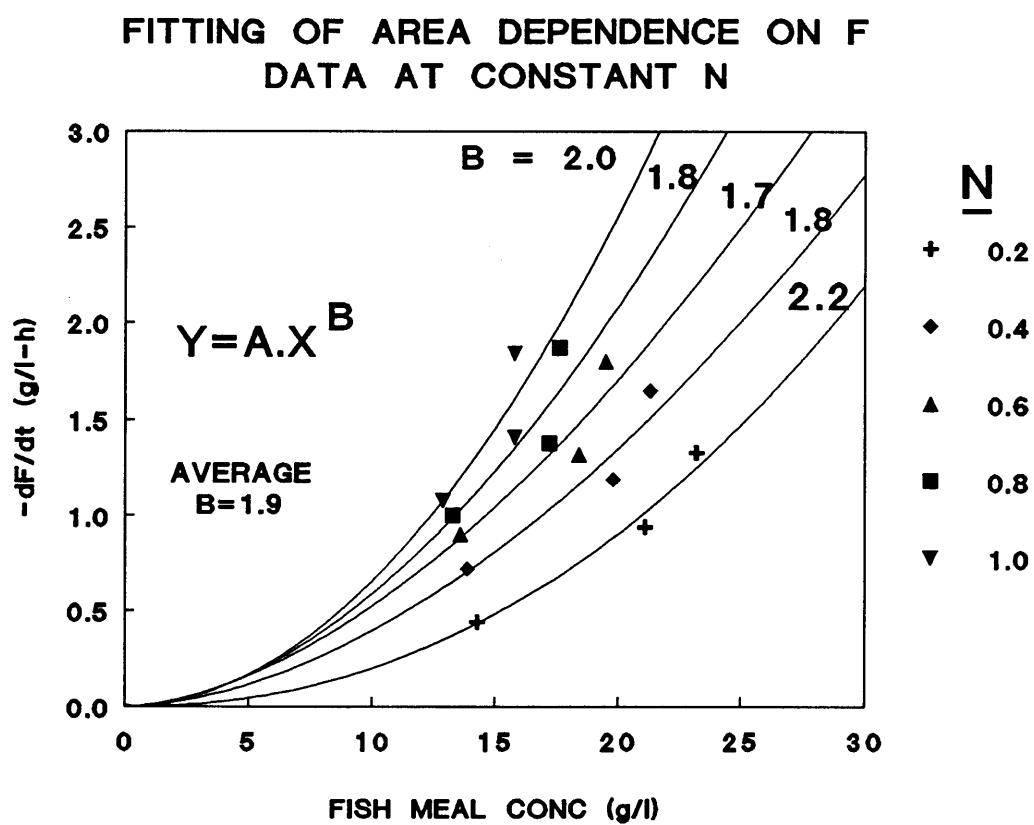


Figure E12: Fitting the parameter B at constant neutral protease concentrations.

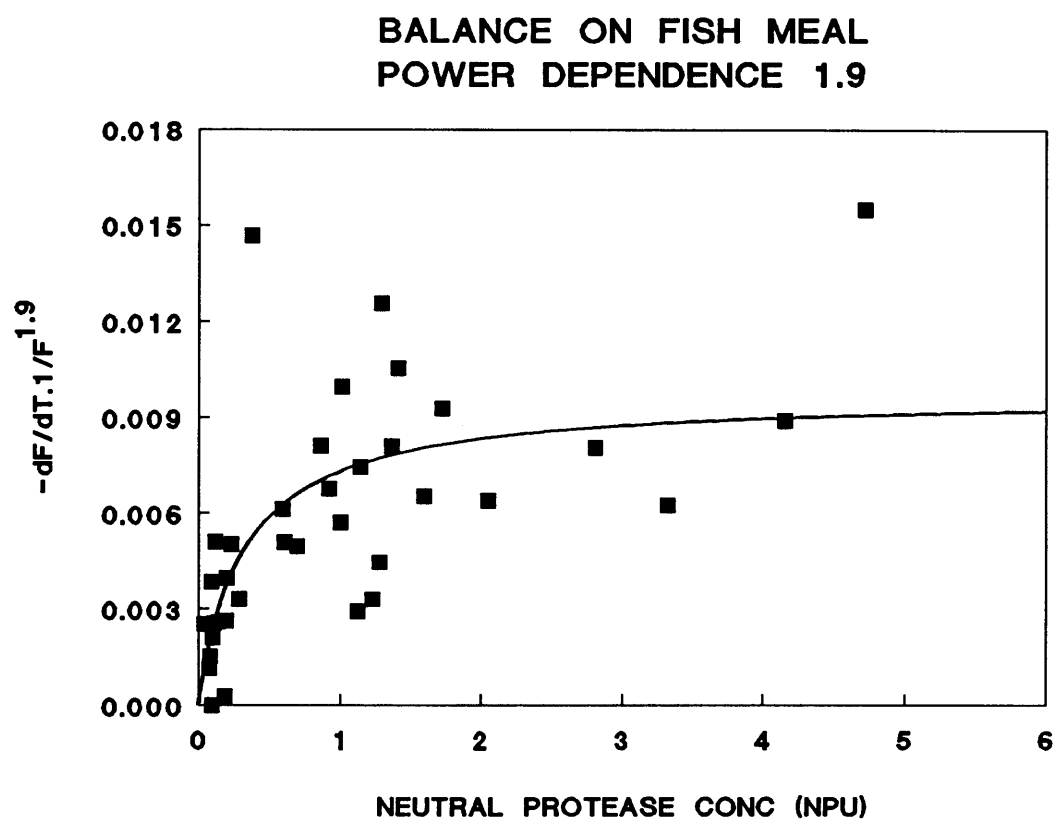


Figure E13: Fitting the parameter K_F using a saturation type plot.

E.8) Balance on Proteases

Alkaline protease accumulation was assumed to occur solely due to formation by the cells. Product destruction was assumed to be negligible. Therefore:

$$\begin{array}{c} \text{ALKALINE} \\ \text{PROTEASE} \\ \text{ACCUMULATION} \end{array} = \begin{array}{c} \text{FORMATION} \\ \text{BY THE} \\ \text{CELLS} \end{array} \quad (\text{E31})$$

$$\frac{dA}{dt} = q_A \cdot X \cdot R_D \cdot R_G \quad (\text{E32})$$

where:

R_D : fishmeal degradation product regulation factor

R_G : glucose regulation factor

q_A : alkaline protease specific productivity (APU.l/g-h)

q_N : neutral protease specific productivity (NPU.l/g-h)

The factors R_D and R_G are used to describe the dissolved fishmeal and glucose regulation of protease production respectively. They are scaling factors that range between 0 and 1. If the factor is 0 protease formation is turned off. If the factor is 1, the cells form protease at the maximum rate as set by the specific productivity and cell concentration. Next an expression was needed to describe these factors as a function of the affecter molecule concentration. Since no relationship could be found in the literature an exponential fit was tested against data that was available in the literature, see figure E14. An exponential curve is a reasonable fit to the data. Therefore:

For dissolved fishmeal and glucose regulation respectively.

$$R_D = \exp(-K_{AD} \cdot D) \quad (\text{E33})$$

$$R_G = \exp(-K_{AG} \cdot G) \quad (\text{E34})$$

where:

$K_{AD}, K_{AG}, K_{ND}, K_{NG}$: constants (1/g)

The specific productivity was modeled using the Ludeking and Piret model, see equation E1. However, since the value $dA/dt \cdot X$ decreased as the dissolved fishmeal decreased during a batch fermentation, see figure E15, q_A must decrease during the fermentation. The only way q_A can decrease during the fermentation is for the specific productivity to be a function of the growth rate which is also decreasing during the fermentation. Thus the β value in equation E1 was set to zero giving:

$$q_A = \alpha_A \cdot \mu \quad (\text{E35})$$

Equation E35 was chosen because mainly because $dA/dt \cdot X$ and growth rate followed the same trend see figure E15. Substituting equations E35, E34 and E33 into equation E32, gives

$$\frac{dA}{dt} = \alpha_A \cdot \mu \cdot X \cdot \exp(-K_{AD} \cdot D) \cdot \exp(-K_{AG} \cdot G) \quad (\text{E36})$$

by analogy the expression for neutral protease production is as follows:

$$\frac{dN}{dt} = \alpha_N \cdot \mu \cdot X \cdot \exp(-K_{ND} \cdot D) \cdot \exp(-K_{NG} \cdot G) \quad (\text{E37})$$

The fit of the data to equations E36 and E37 can be seen in figures E16 and E17 respectively, and can be best represented as three dimensional plots. These plots indicate that the higher the glucose and dissolved fishmeal concentrations the less protease is produced. There is considerable scatter in these plots.

Equation E17 was used to predict the dissolved fishmeal concentration shown in figures E16 and E17 in all fermentations except one. In the control experiment, the dissolved fishmeal was estimated using the model as rapid addition of fishmeal did not permit dF/dt to be determined. In a small number of cases the glucose was estimated based on carbon dioxide evolution data.

To get the best representation of the regulation effects, over as wide a range of glucose and dissolved fishmeal concentrations as possible, data from five fermentations, including the batch, fed-batch and control fermentations were incorporated into figures E16 and E17 and hence the determined parameters.

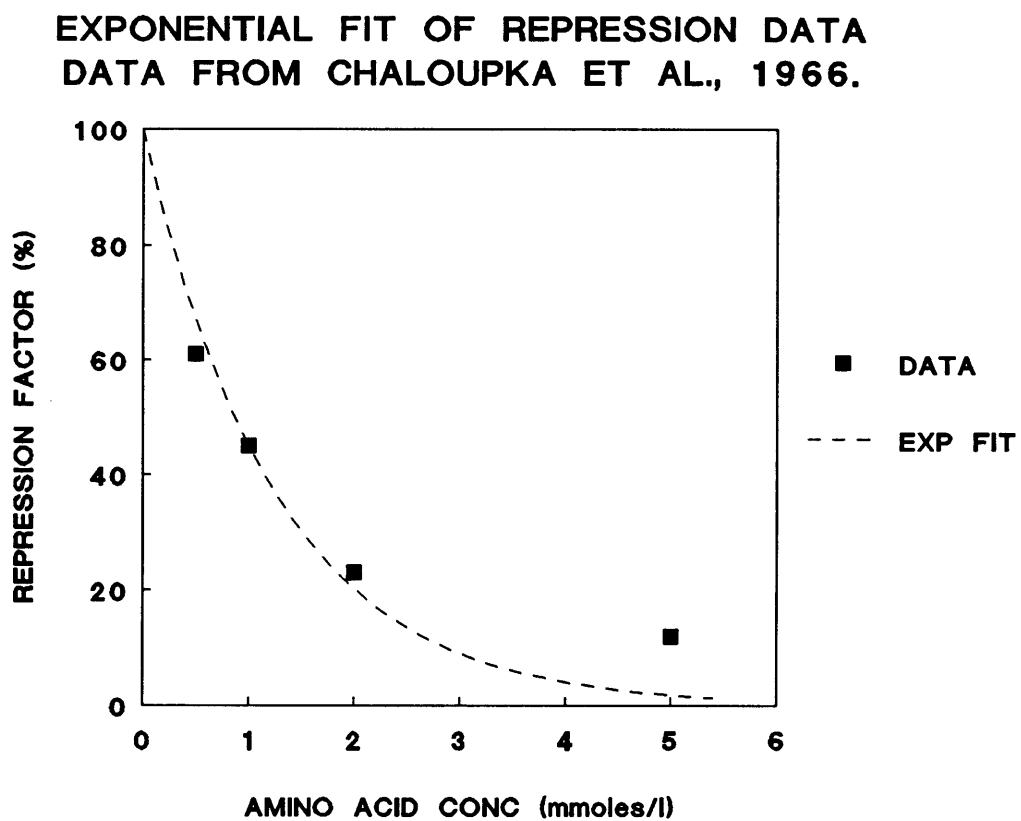


Figure E14: An exponential fit to data showing the effect of amino acid concentration on protease formation. Data from Chaloupka et al., 1966.

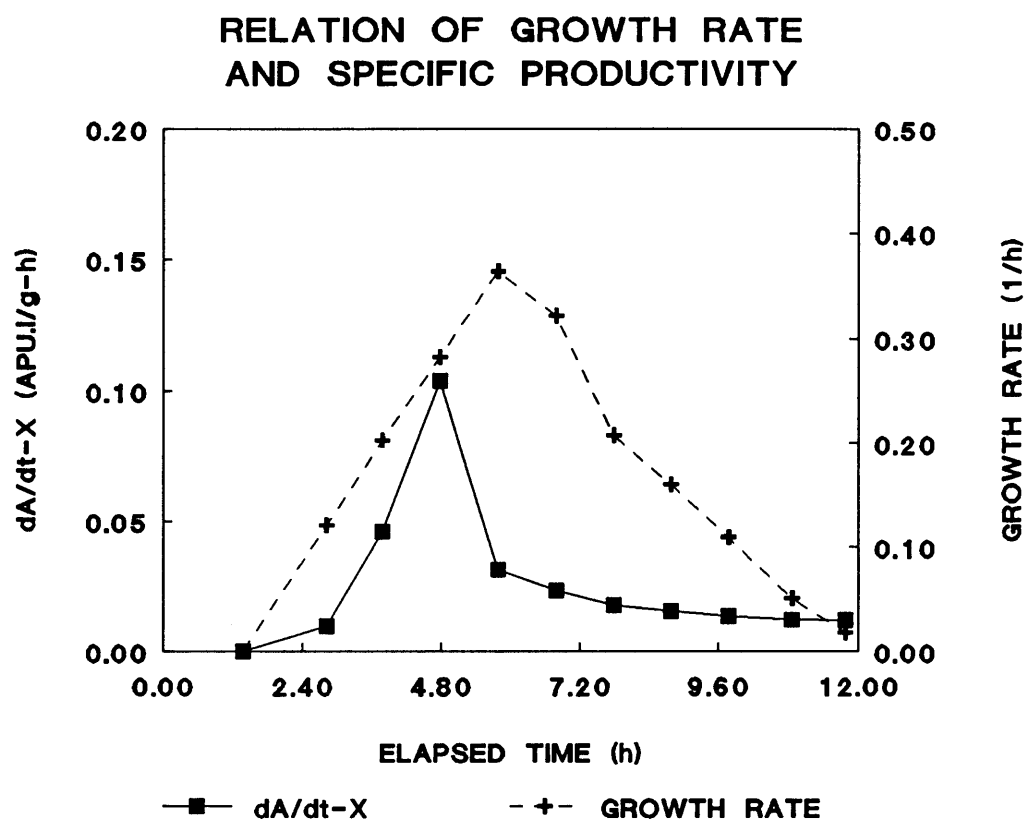


Figure E15: The change in $dA/dt-X$ and growth rate with time during the batch fermentation on solid substrate.

ALKALINE PROTEASE

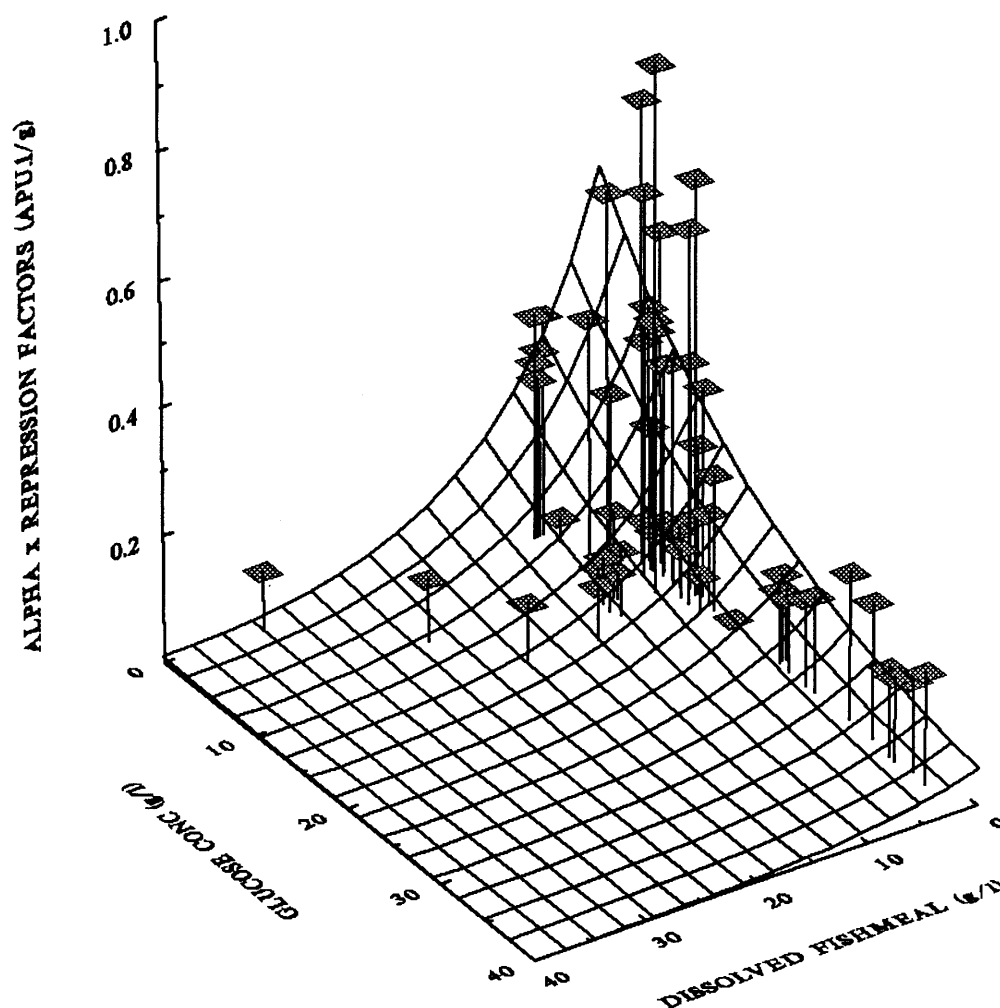


Figure E16: Fit of the alkaline protease repression data to equation E36. The squares represent the data points, the vertical lines show the location of the data points above the base plane and the curved surface indicates the model, equation E36.

NEUTRAL PROTEASE

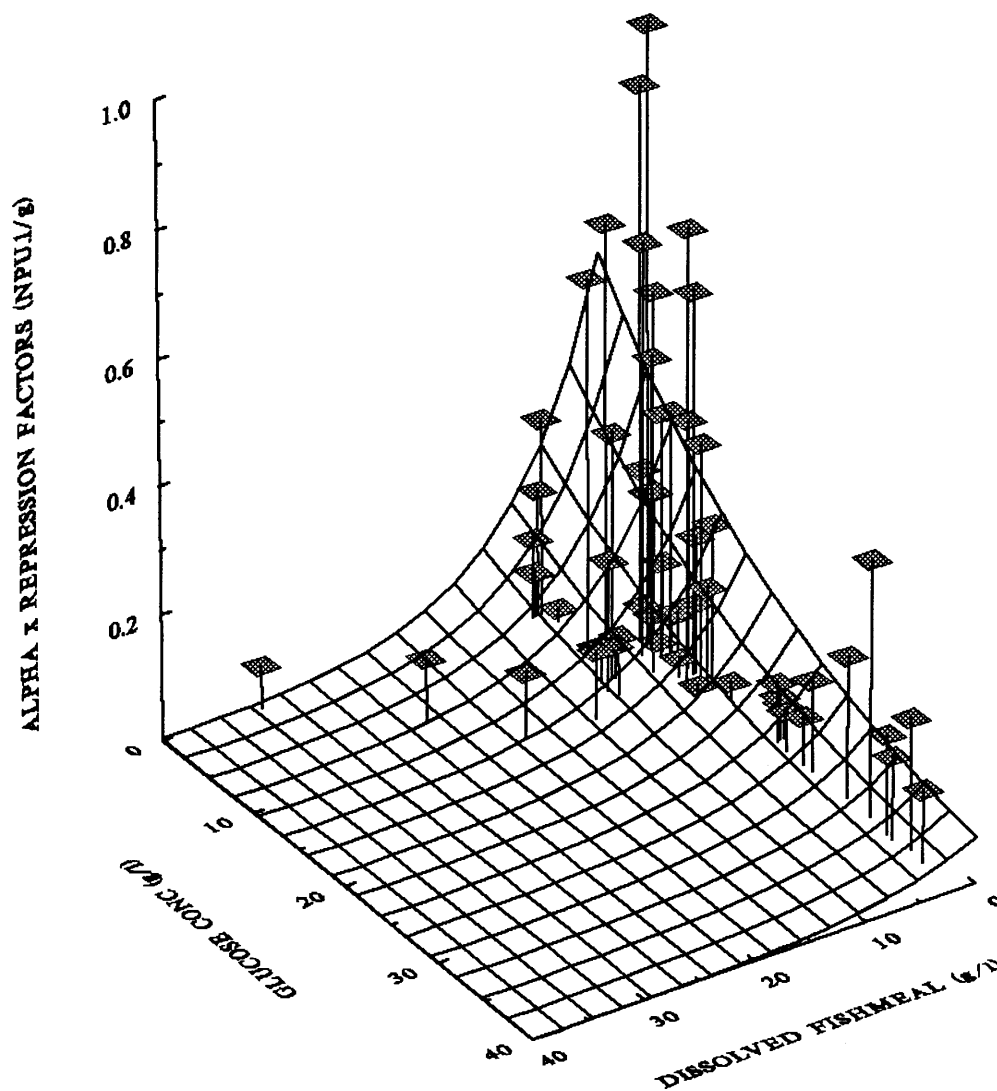


Figure E17: Fit of the neutral protease repression data to equation E37. The squares represent the data points, the vertical lines show the location of the data points above the base plane and the curved surface indicates the model, equation E37.

E.9) Fit of the Model to the Batch and Fed-Batch Fermentations

Once the original form of the equations was developed from mainly the batch and fed-batch fermentation data, these fermentations were modelled to check how well the model fits the data from which it was derived. The results for the batch fermentation can be seen in figures E18 to E21. The results for the fed-batch fermentation can be seen in figures E22 to E25. The model actually used to produce figures E18 to E25 incorporated the effect of toxic product formation but since only a small amount of toxic product was formed in these fermentations its effect was negligible. The BASIC program used to generate the model predictions can be seen in appendix F.

The model fitted the data with the exception of the predicted glucose profile, which deviates from the data in both fermentations. The most likely explanation for the deviations in the glucose profile was that the total glucose concentration added and consumed was much larger than the glucose concentration in the fermentor. Thus the glucose concentration is determined as the difference between two large numbers.

Neutral protease profiles are not shown because they are almost identical to the alkaline protease profiles.

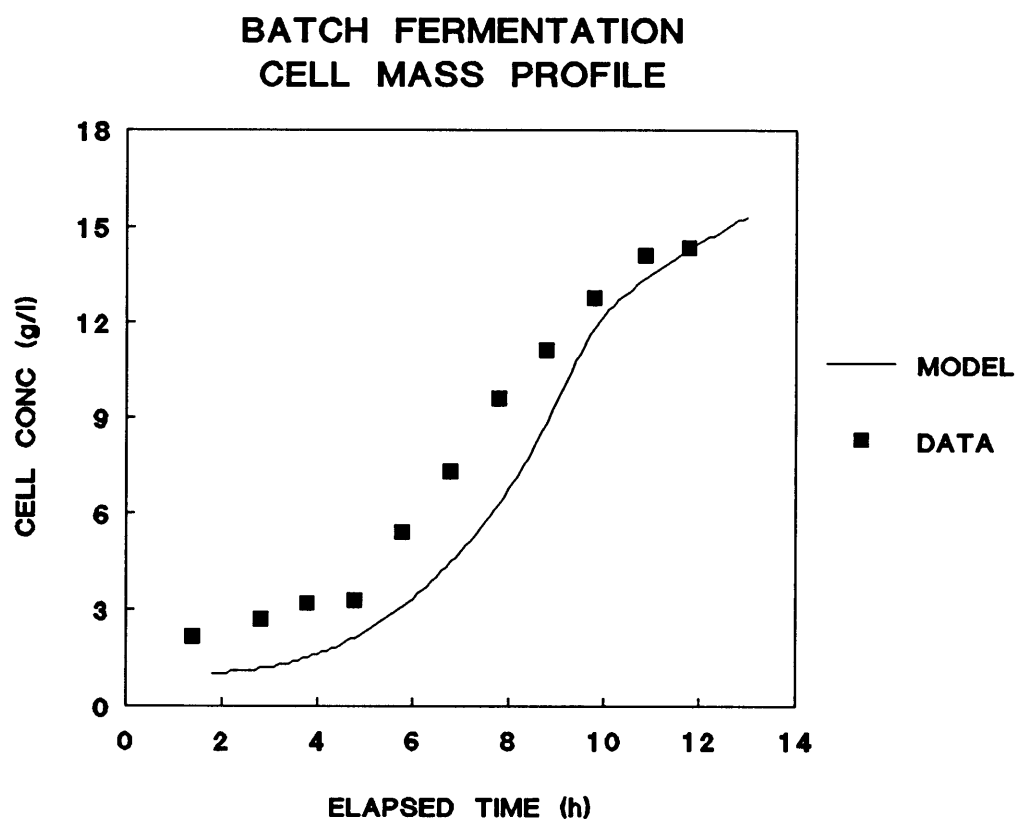


Figure E18: Cell mass profile during the batch fermentation.

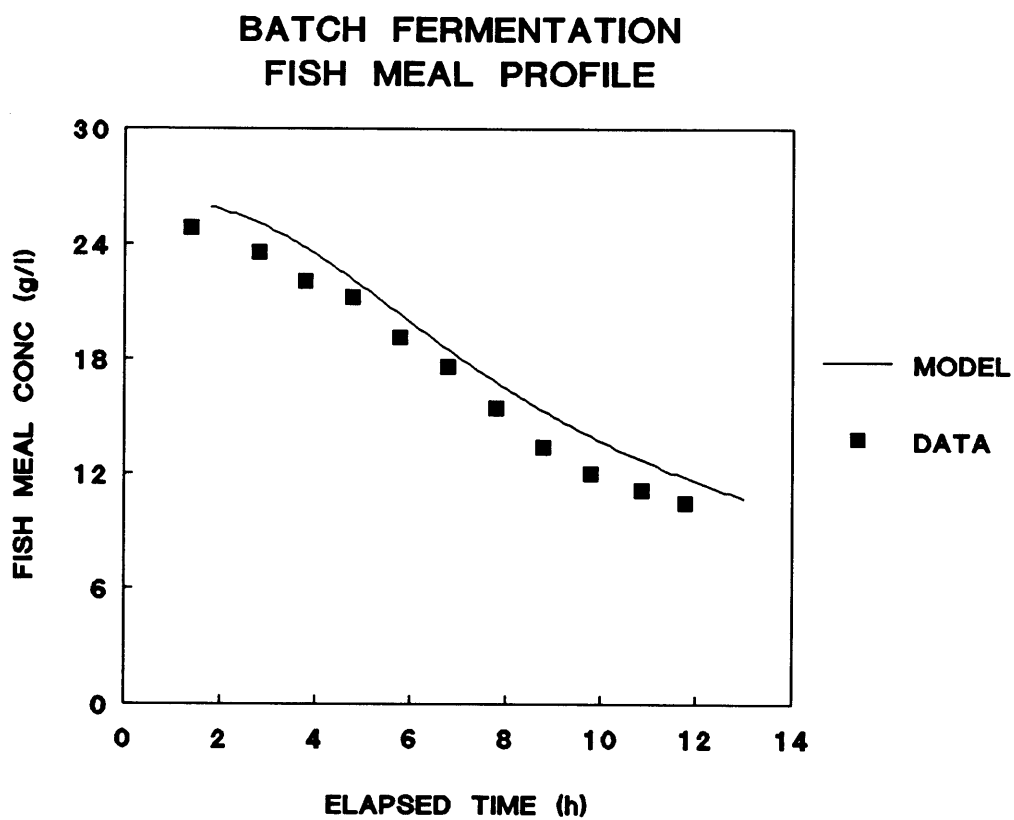


Figure E19: Fishmeal profile during the batch fermentation.

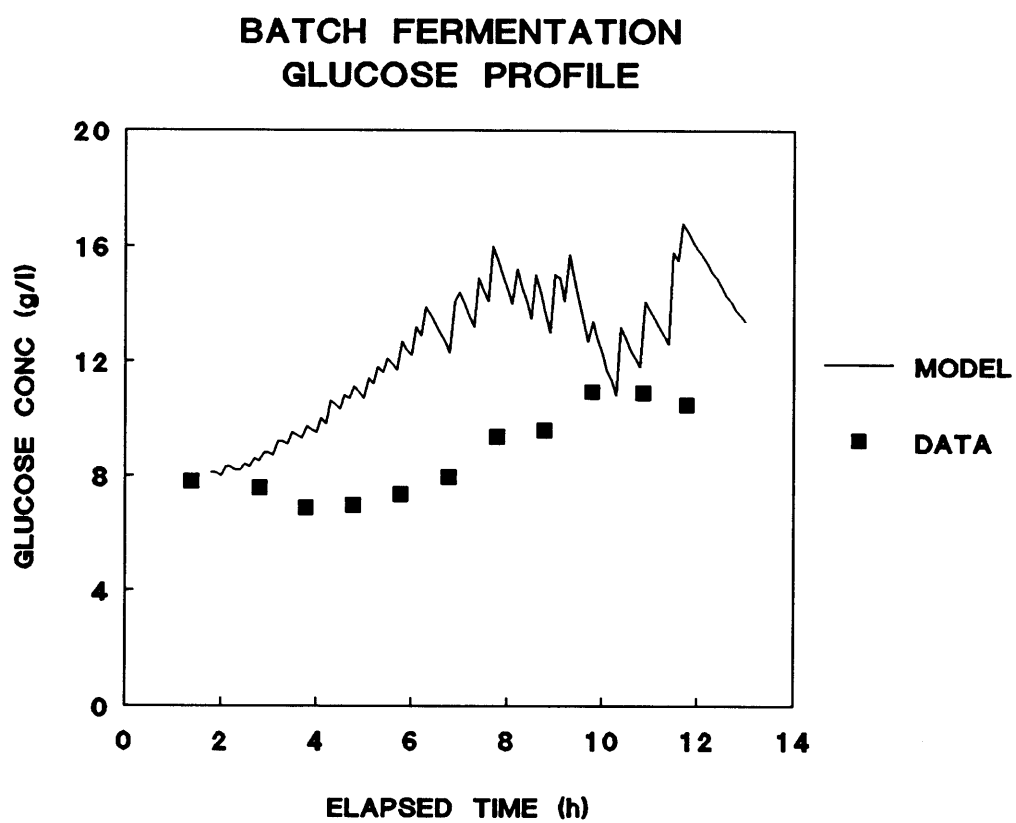


Figure E20: Glucose profile during the batch fermentation.

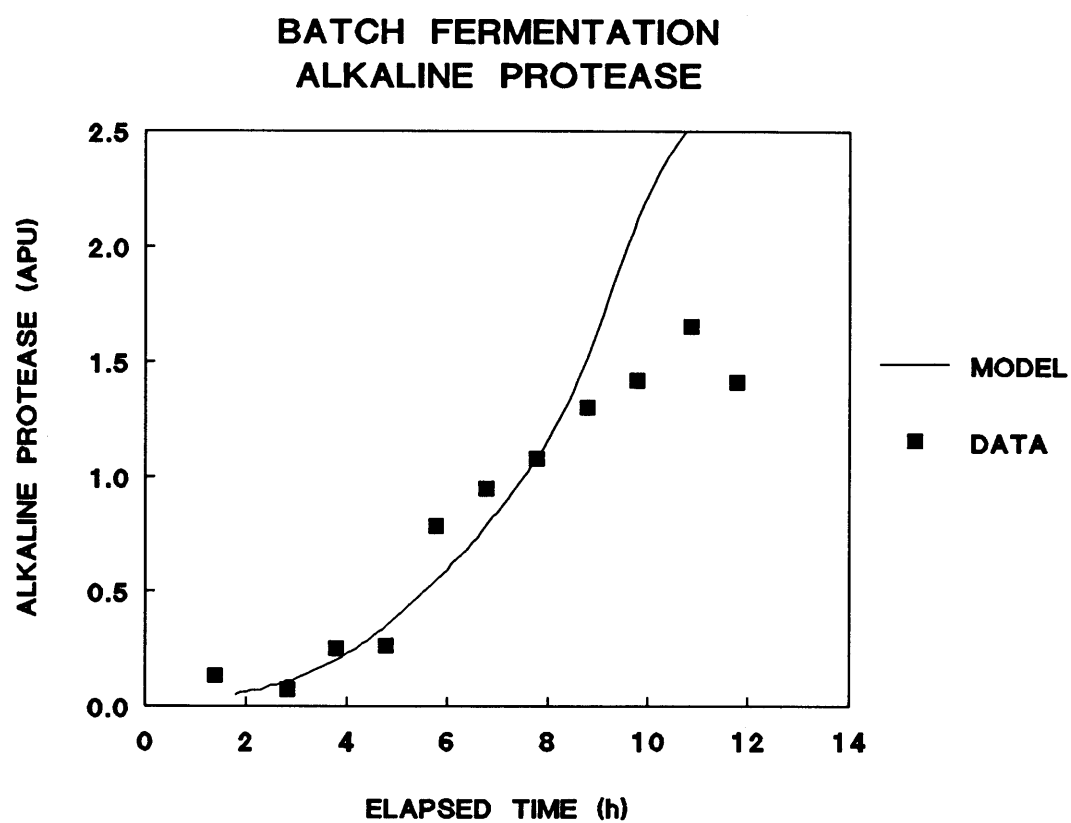


Figure E21: Alkaline protease profile during the batch fermentation.

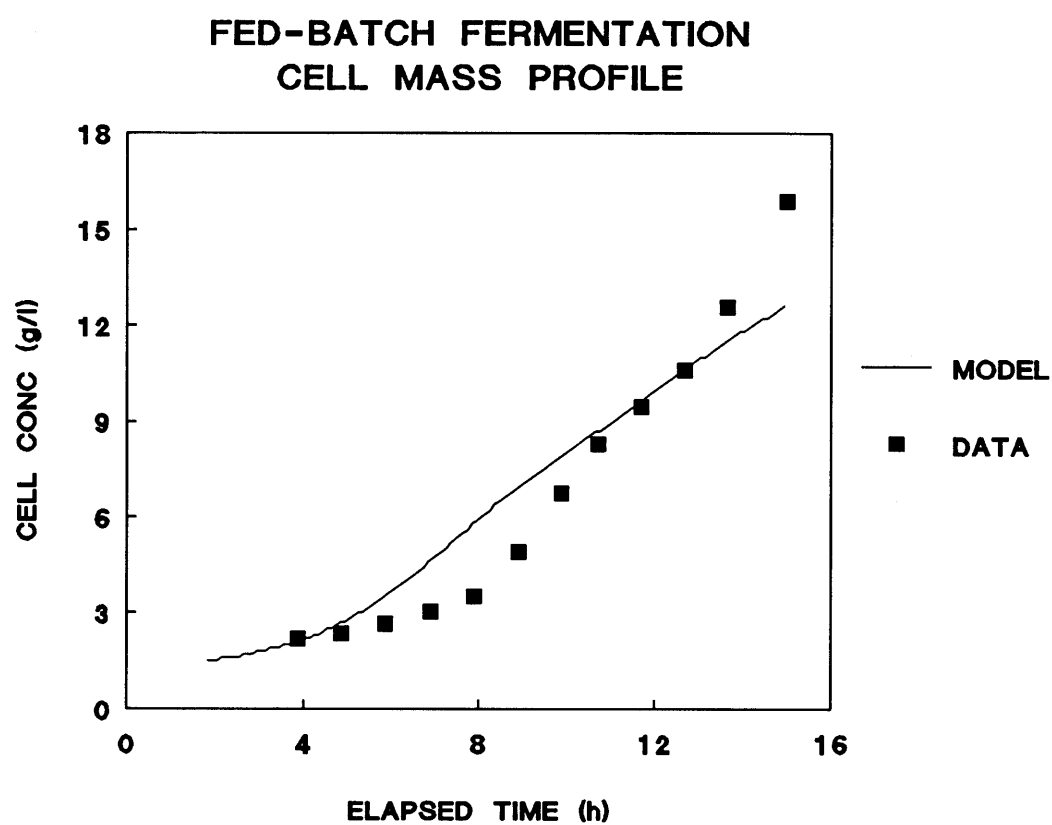


Figure E22: Cell concentration profile during the fed-batch fermentation.

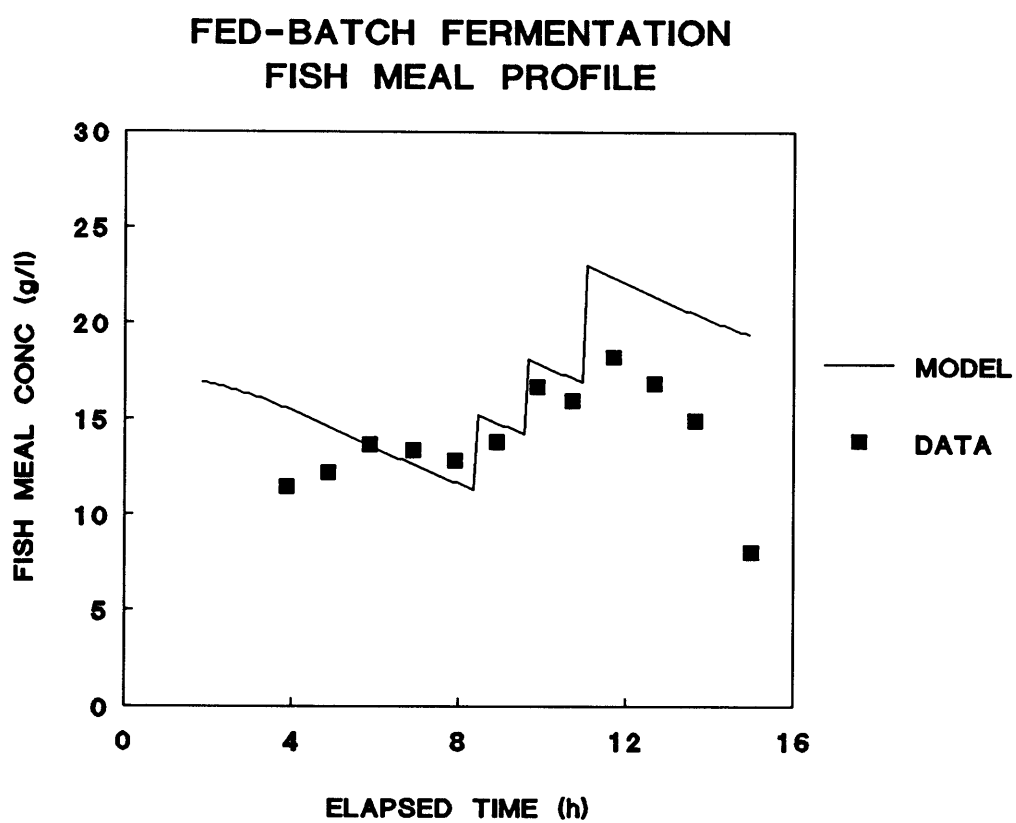


Figure E23: Fishmeal profile during the fed-batch fermentation.

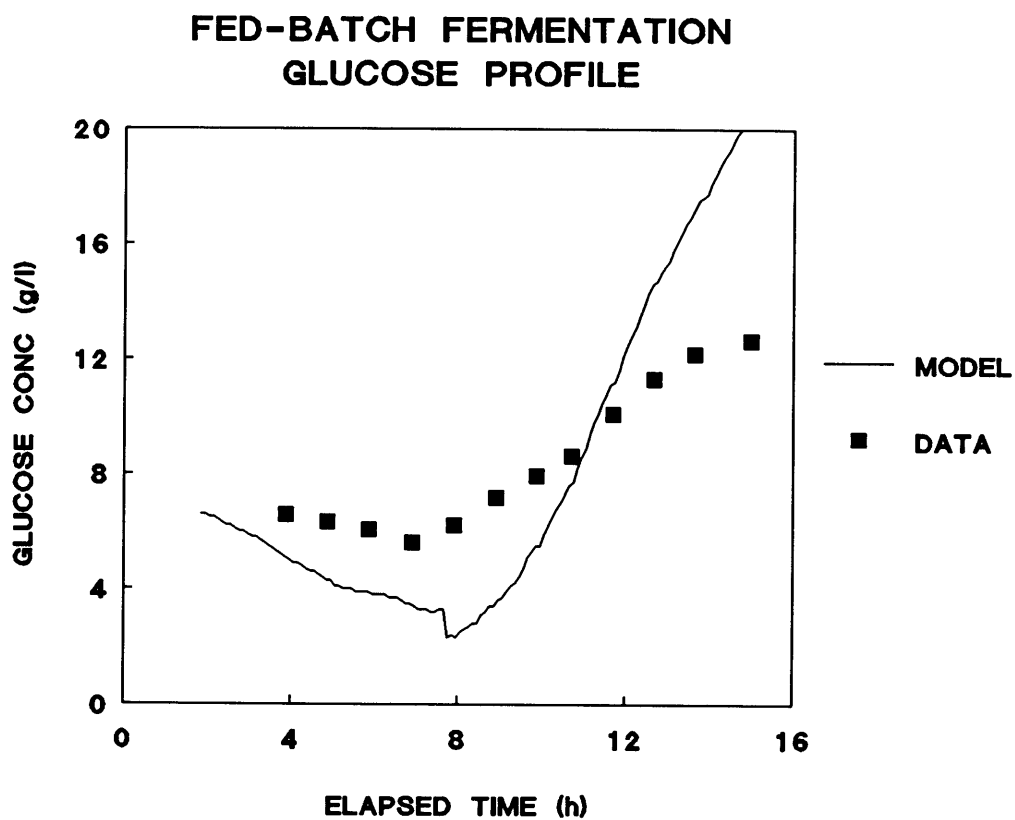


Figure E24: Glucose profile during the fed-batch fermentation.

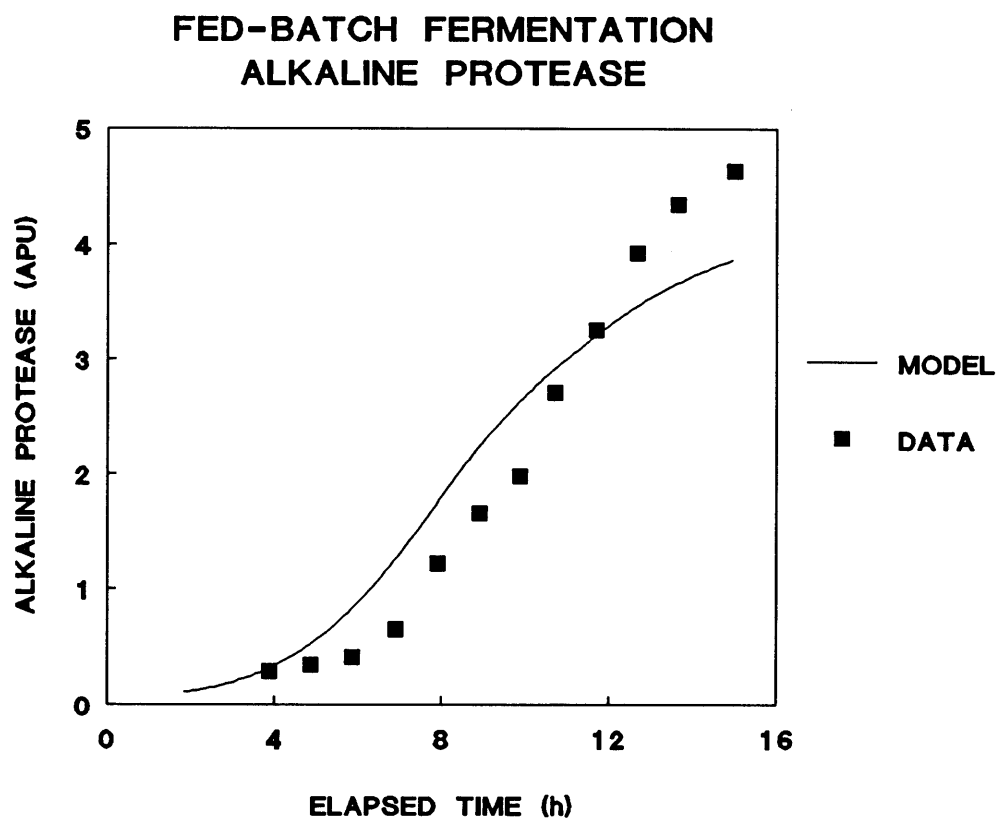


Figure E25: Alkaline protease profile during the fed-batch fermentation.

E.10) Extension of the Model and a Control Experiment.

Now that a model had been developed it was used to try to improve alkaline protease volumetric productivity by controlling the dissolved fishmeal concentration. The objectives of the control fermentation were as follows:

- 1) To test the model. Does it correctly predict the time course of the fermentation?
- 2) To optimize the model (the objective function is to maximize the alkaline protease volumetric productivity). The results of the optimization, along with on-line data, were used to feed fishmeal during the control experiment.
- 3) To identify a hierarchy of limitations that prevent maximum protease formation from being achieved.

The model was optimized as it was (without the effect of toxic product formation which was unknown at the time of the optimization). The model was optimized with respect to fishmeal addition by my colleague Seujeung Park to maximize alkaline protease specific productivity for a given fermentation time. The glucose profile was not optimized but assumed constant at 8.7 g/l throughout the fermentation. This was because the major interest in this study was solid substrate utilization and not glucose utilization. The program used to optimize the model can be seen in appendix G.

Maximizing the specific productivity for a fixed fermentation time was chosen as a measure of the maximum alkaline protease volumetric productivity because the optimization results stated that there was no optimal length of time to run the fermentation. The optimization results stated that the longer you could run the fermentation the greater the

volumetric productivity would be. Since the model did not include other factors that would eventually halt the fermentation, such as the reactor filling up or oxygen limitation, it was decided to optimize a 12 hour fermentation. Twelve hours was chosen because the model predicted that at this point that the fermentation parameters would have reasonable values.

It was also decided that the fermentation would be kept running for as long as possible after 12 hours to see what limitations occurred and how much protease could be accumulated. The model was again used to predict the fishmeal addition rate which would keep the dissolved fishmeal concentration at 2.0 g/l which is the predicted value of dissolved fishmeal at the end of the 12 hour optimal fermentation. The program used to determine the fishmeal feed profile after 12 hours can be seen in appendix H.

The model was optimized as a constant volume fermentation because the volume of glucose and fishmeal added was very low and balanced the volume taken from the fermentation as samples. Although a constant volume was not achieved in practice it was a reasonable assumption. The profiles predicted by the optimization of the model for fishmeal addition, dissolved fishmeal, cell, solid and alkaline protease concentrations can be seen in figures E26-E29.

The control experiment was then performed based on the model optimization results and on-line measurements. The on-line estimate of the dissolved fishmeal concentration was used to correct the model predicted fishmeal addition rate. Since the growth rate was known the dissolved fishmeal concentration was estimated on-line using equation E12. The optimization results were corrected by on-line estimates because of the difficulty in estimating the initial conditions at the beginning of the fermentation and also because the

scatter in the model correlations suggested that considerable error may be present in the model parameters.

The fishmeal was added every 15 minutes during the fermentation. Fishmeal was added manually through the top of the fermentor. A large number of conical flasks containing different amounts of fishmeal were prepared and autoclaved prior to the fermentation. The amount of fishmeal to be added could be determined by either the model, or by the model combined with growth rate data from either the light scatter technique's or the carbon dioxide evolution technique's estimate of cell concentration. When the amount to be added was determined then the conical flask containing the closest amount of fishmeal to that required was selected and that amount of fishmeal added to the fermentor. The exact amount of fishmeal added was recorded and entered into Paragon.

The amount of fishmeal to be added based on the model optimization results was modified on-line using a PID controller. The model optimization results were modified based on growth rate data. The growth rate data was used to calculate a limiting dissolved fishmeal concentration, assuming a Monod type relationship between growth rate and dissolved fishmeal concentration. If the estimated dissolved fishmeal concentration was different from the model predicted dissolved fishmeal concentration then the amount of fishmeal to be added specified by the model was adjusted as follows:

$$ERR = (DFM_{model} - DFM_{\mu}) \quad (E38)$$

where

- ERR : Error in dissolved fishmeal concentration between the model estimate and the growth rate estimate (g/l).
 DFM_{model} : Dissolved fishmeal concentration predicted by the model optimization (g/l).
 DFM _{μ} : Dissolved fishmeal concentration based on growth rate data (g/l).
 and

$$C_A = M_{PRED} + K_{PR} \cdot ERR + K_{INT} \sum_1^{N_C} ERR \cdot \Delta t_F + K_{DR} \cdot \frac{dERR}{dt} \quad (E39)$$

where

- C_A : Control action, the amount of fishmeal to add during a 15 minute interval (g/l).
 M_{PRED} : Model predicted amount of fishmeal to add during a 15 minute interval (g/l).
 K_{PR} : Proportional control constant (g fishmeal/g dissolved fishmeal).
 K_{INT} : Integral control constant (g fishmeal/g dissolved fishmeal.h)
 K_{DR} : Derivative control constant (g fishmeal.h/g dissolved fishmeal).
 N_C : The total number of fishmeal feed scans to date
 Δt_F : The time between fishmeal feed scans

The PID control parameters, K_{PR}, K_{DR} and K_{INT} were determined based on perturbations to the model. A perturbed model was developed in which each parameter in the model was underestimated by 20%. The perturbed model gave an estimate of the dissolved fishmeal that was different to that predicted by the model itself. The control parameters were then tuned until the control action returned the perturbed model estimate of the dissolved fishmeal back to the actual model estimates of the dissolved fishmeal concentration. The FORTRAN program used to tune the PID control parameters can be

seen in appendix I. The obtained PID control parameters are as follows:

K_{PR} : 3.16 g fishmeal/g dissolved fishmeal

K_{INT} : 5.80 g fishmeal/g dissolved fishmeal-h

K_{DR} : 0.40 g fishmeal.h/g dissolved fishmeal

The performance of these PID parameters in returning the perturbed model estimates of the dissolved fishmeal concentration back to the actual model estimates of the dissolved fishmeal concentration can be seen in figure E30. In the control fermentation the above parameter values were used except a K_{INT} value of 1.16 g fishmeal/g dissolved fishmeal-h was used. This lower value of K_{INT} was used inadvertently.

The control experiment immediately exposed a deficiency in the model. Towards the end of the fermentation the dissolved fishmeal was predicted to be approximately 24 g/l and the glucose concentration was 12 g/l. These values predicted a growth rate of 0.7 1/h according to the model. However, the actual measured growth rate was approximately 0.15 1/h. This meant that the on-line estimate of dissolved fishmeal concentration was too low compared to the model and the PID controller kept on adding fishmeal to increase the growth rate. When the growth rate would not respond to the additions of fishmeal the PID controller would add even more fishmeal to try to increase the growth rate because of the integral term in the PID controller. This meant that the fermentation got swamped with dissolved fishmeal but the growth rate remained low. Something else and not dissolved fishmeal was limiting the growth of the culture.

The excess dissolved fishmeal present in the fermentation medium meant that negative regulation occurred and that the protease titer in the control experiment was low. This points to a weakness in modeling approaches to the control of fermentations. The models are based on data obtained to date. But if that data does not represent a wide range of conditions, or inaccurately represents the data, then the model is likely to fail. Models can be very successful retrospectively but as they are extended problems can arise.

Toxic products were suspected to be responsible for limiting the growth of the culture. In order to test this hypothesis the HPLC was used to detect the presence of end products. Also since end products effects were not noticed in the batch and fed-batch fermentation they should be absent or in low concentrations in these fermentations. The results of the HPLC analysis can be seen in figures E31 and E32. Three main end products were identified in the control experiment, acetate, acetoin and 2,3-butanediol. In the batch and fed-batch fermentation these compounds were present in only small quantities compared to the concentrations observed in the control experiment. Acetate and acetoin were present in the highest concentrations and their profiles for the three fermentations can be seen in figures E33 and E34.

Next since end products had been identified as being present we had to determine if they were indeed toxic to organism growth. The literature provided numerous examples of acetate and acetoin being toxic to cell growth at the concentrations observed in the control experiment, see section 2.3.3. Also the literature showed that the growth rate was linearly correlated to toxic product concentration for these two chemicals. Thus the effect of toxic product concentration on the growth rate was modeled as follows.

$$\mu = (T_1 - T_2 A_E) \cdot (T_1 - T_3 A_N) \quad (\text{E40})$$

where:

A_E : acetate concentration (g/l)

A_N : acetoin concentration (g/l)

T_1 : constant (1/h)

T_2 : constant (1/g-h^{0.5})

T_3 : constant (1/g-h^{0.5})

The data used to fit this correlation came from the control experiment and included only those data in which the growth rate was lower than that expected based on the predicted dissolved fishmeal concentration. The fit of the model to the data can be seen in figure E35. This three dimensional plot relates the growth rate to the acetate and the acetoin concentrations. Since it cannot be easily seen how well the data fits the model in this sort of plot the data is replotted in two dimensional space in figure E36. Figure E36 is an unusual plot. In this plot the growth rate is represented by the size of the radius of the symbol. Thus the effect of acetate and acetoin on the growth rate can be easily viewed by the symbol size. More importantly the plot shows how well the model and the data overlap. If the model and the data match then the two symbols should be the same size. A discrepancy in symbol size between the model and the data indicates a poor fit. As can be seen in figure E36 a good fit to the data was obtained.

The new model, taking into account the effect of the toxic products on growth rate, was then fitted to the control experiment. The results can be seen in figures E37 to E40. The model matches the data with the exception of the glucose profile. The causes of the poor match to the glucose profile, especially at the end of the fermentation are likely to be

the same as those responsible for the similar discrepancy in the batch and fed-batch fermentations, see section E.9.

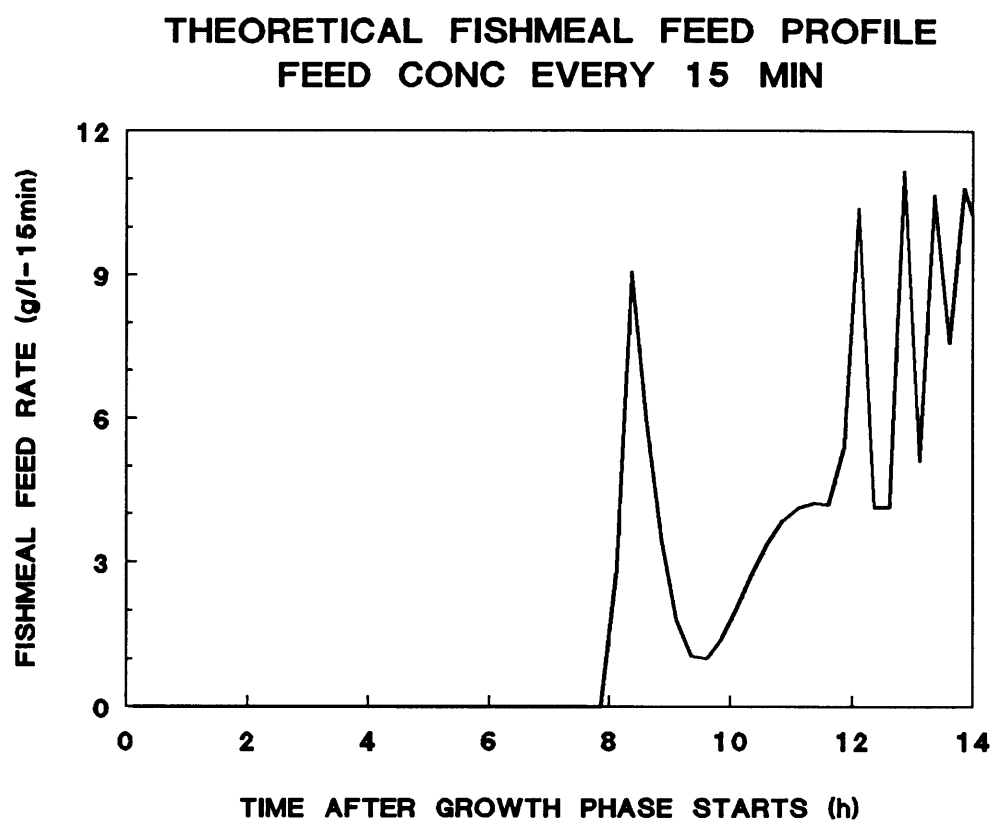


Figure E26: The fishmeal addition rate predicted by the model optimization.

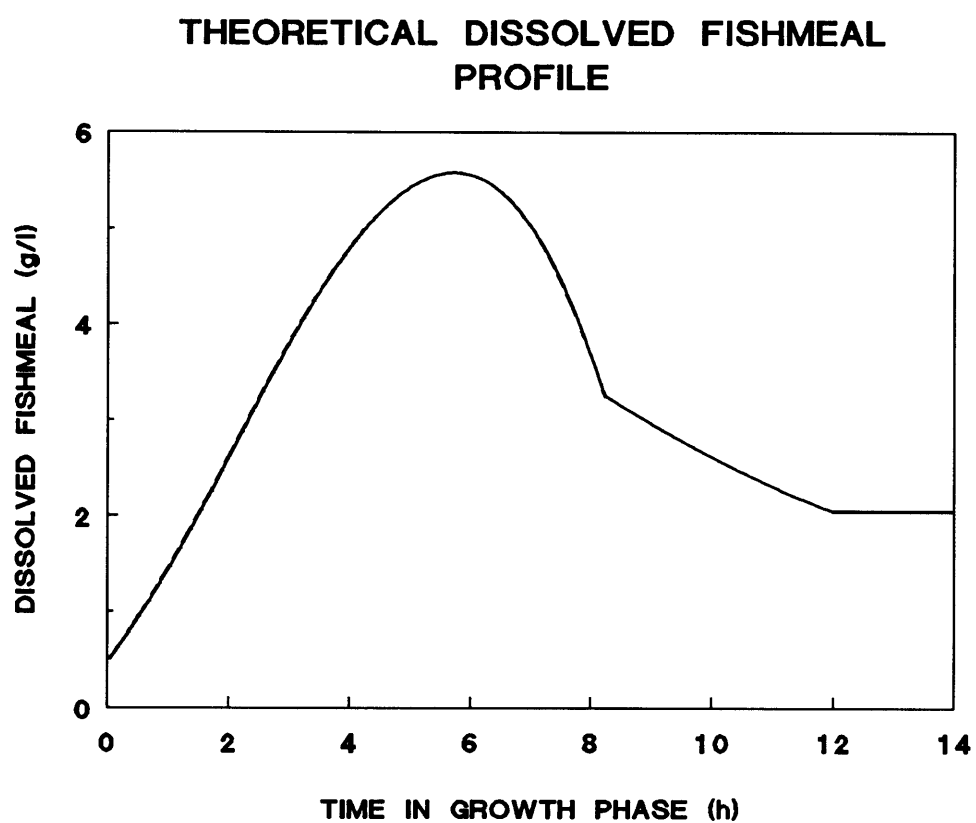


Figure E27: The dissolved fishmeal profile predicted by the model optimization.

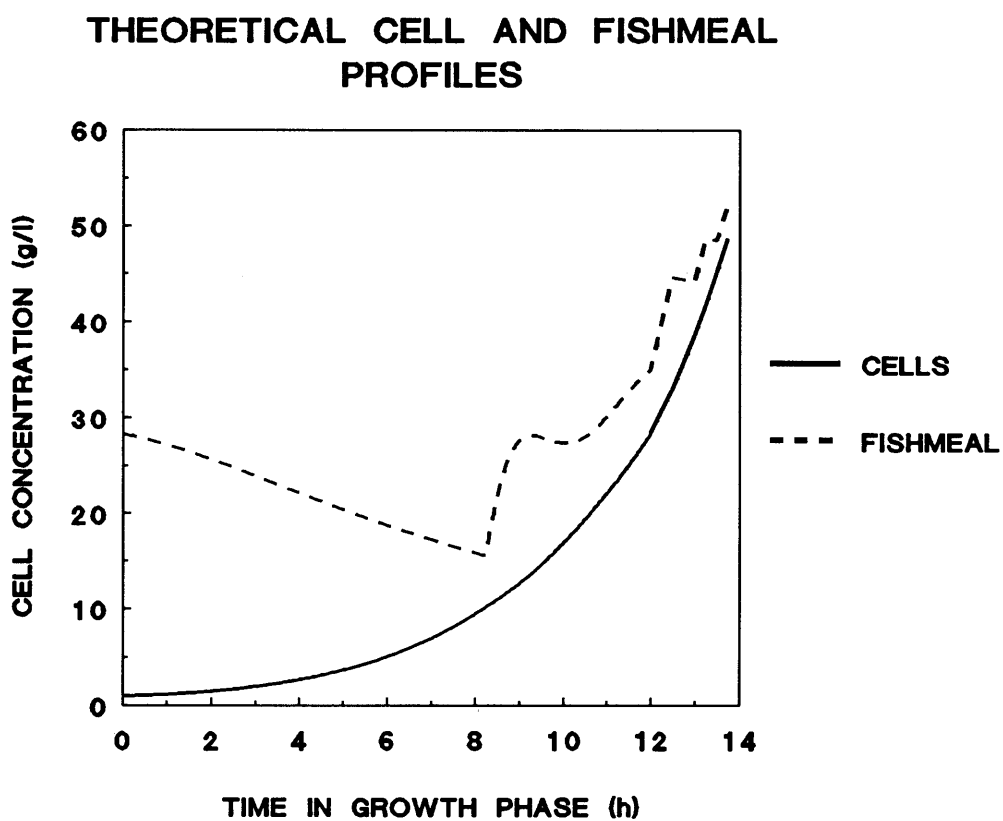


Figure E28: The cell and fishmeal concentration profile predicted by the model optimization.

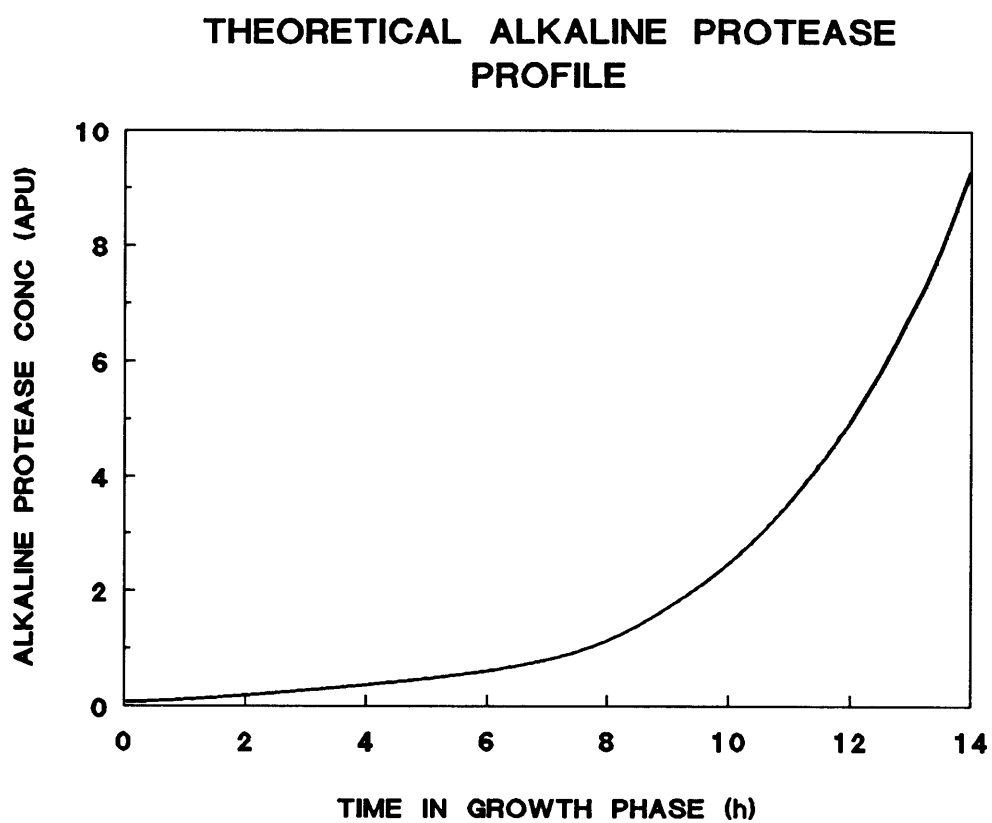


Figure E29: The alkaline protease activity predicted by the model optimization.

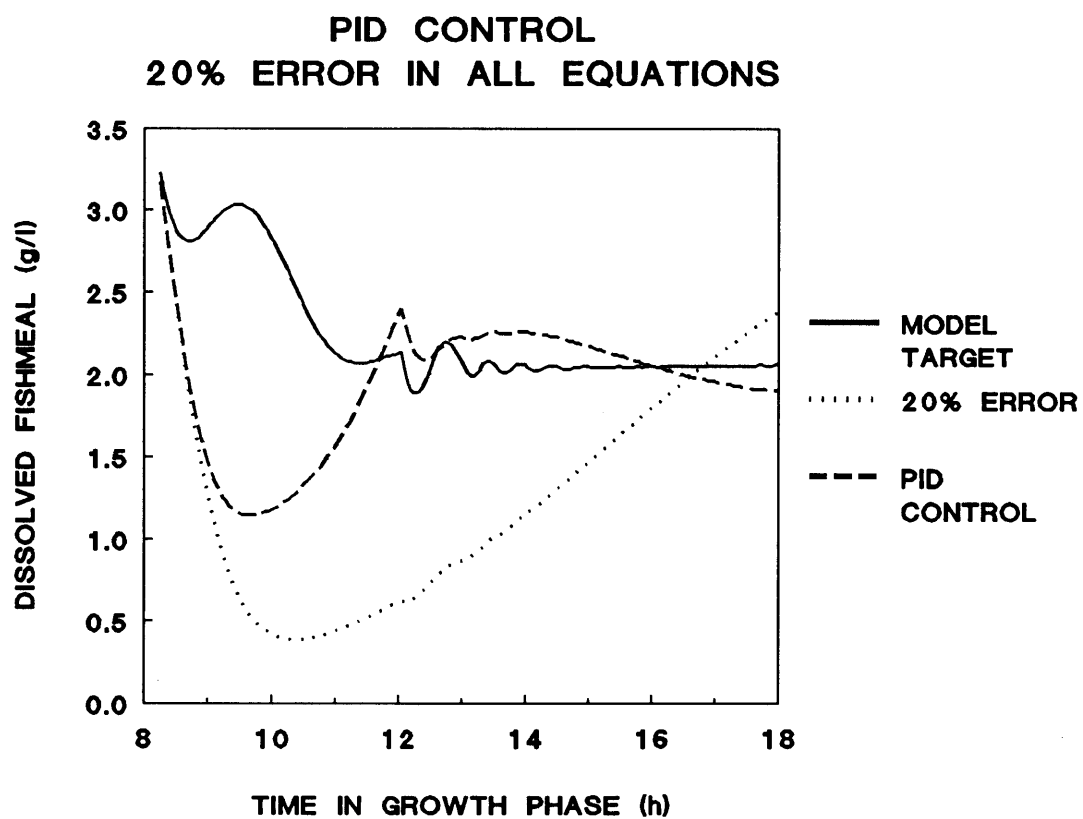


Figure E30: The performance of the PID control parameters in returning the perturbed model estimates of the dissolved fishmeal to those predicted by the actual model.

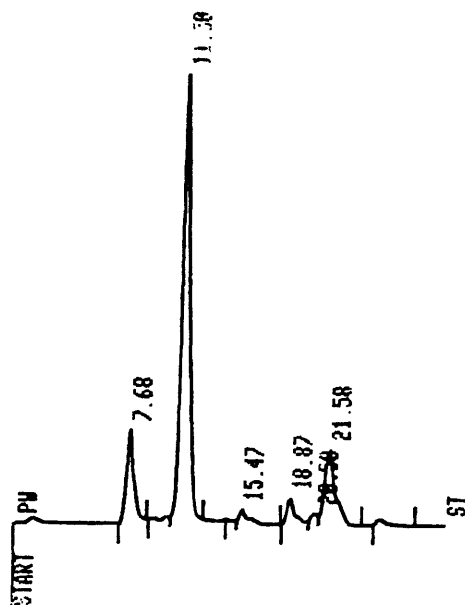


Figure E31: The HPLC results from a sample taken during the fed-batch fermentation.

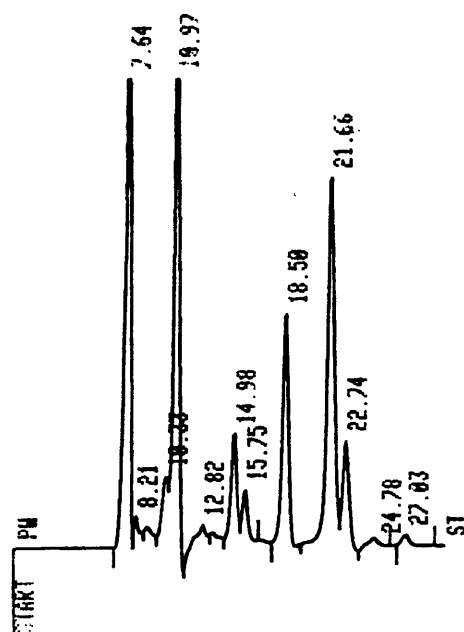


Figure E32: The HPLC results from a sample taken during the control fermentation.

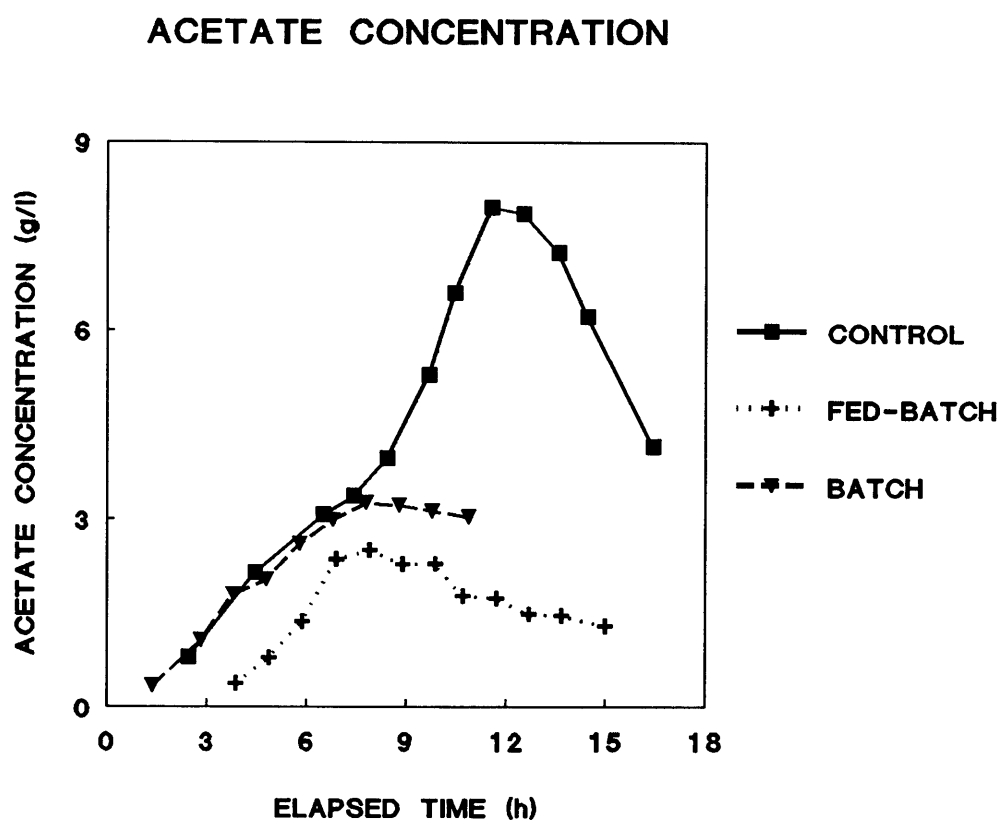


Figure E33: The acetate concentration profiles of the batch, fed-batch and control fermentations.

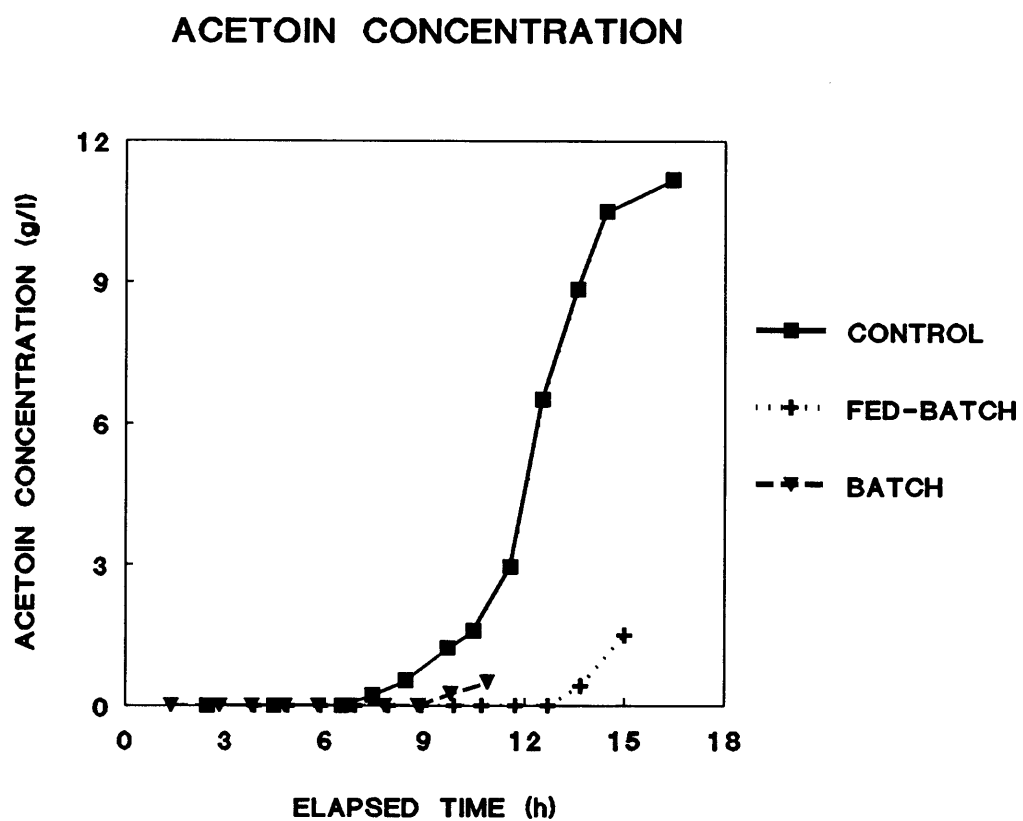


Figure E34: The acetoin concentration profiles from the batch, fed-batch and control experiments.

EFFECT ON GROWTH RATE *ACETATE AND ACETOIN*

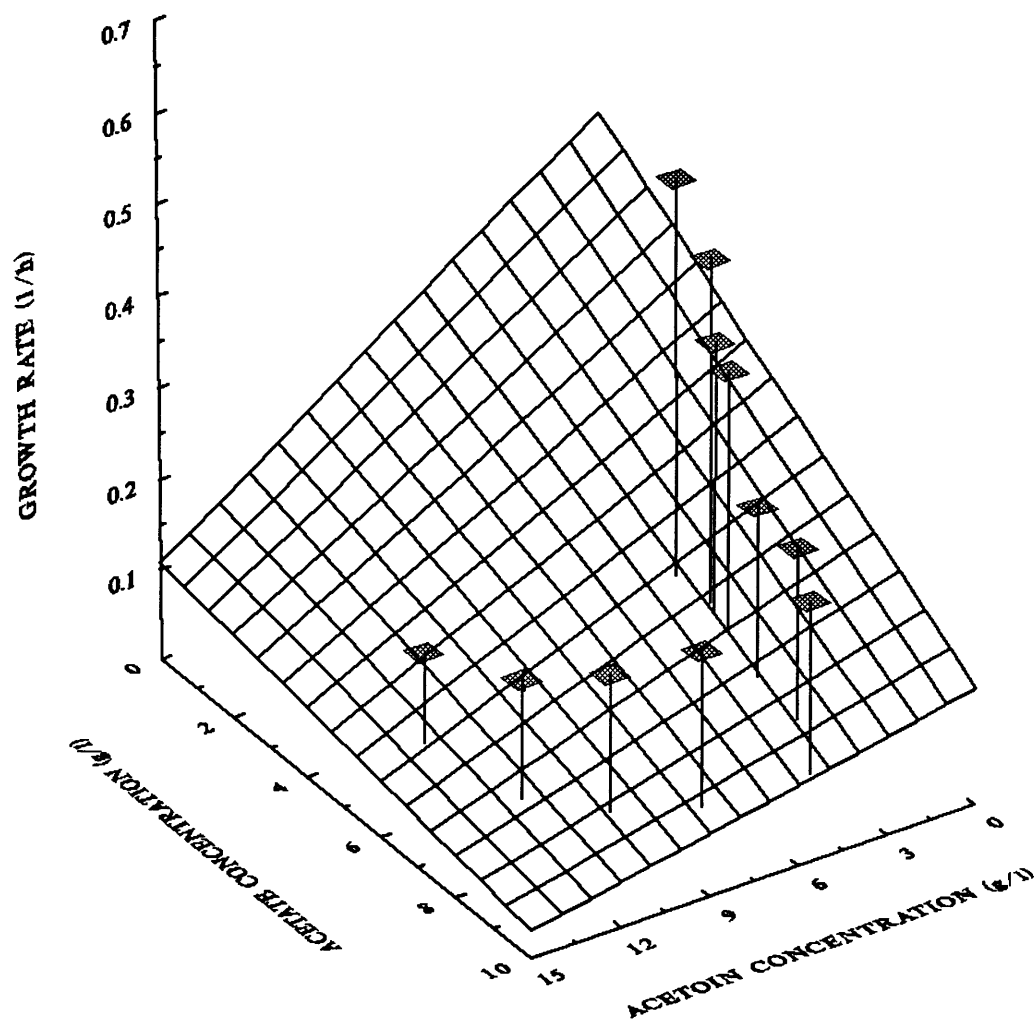


Figure E35: The effect of acetate and acetoin on the growth rate, three dimensional representation.

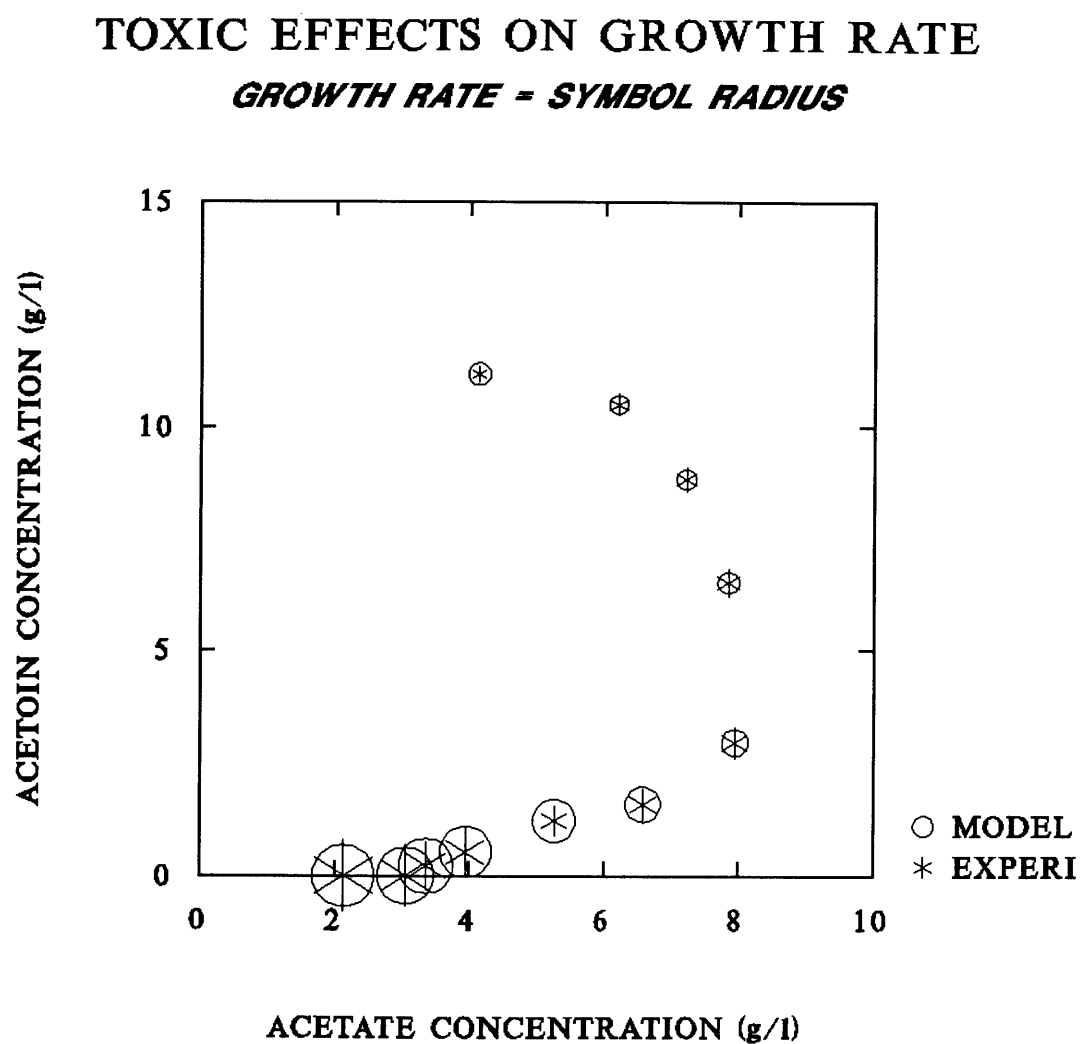


Figure E36: The effect of acetate and acetoin on the growth rate, two dimensional representation.

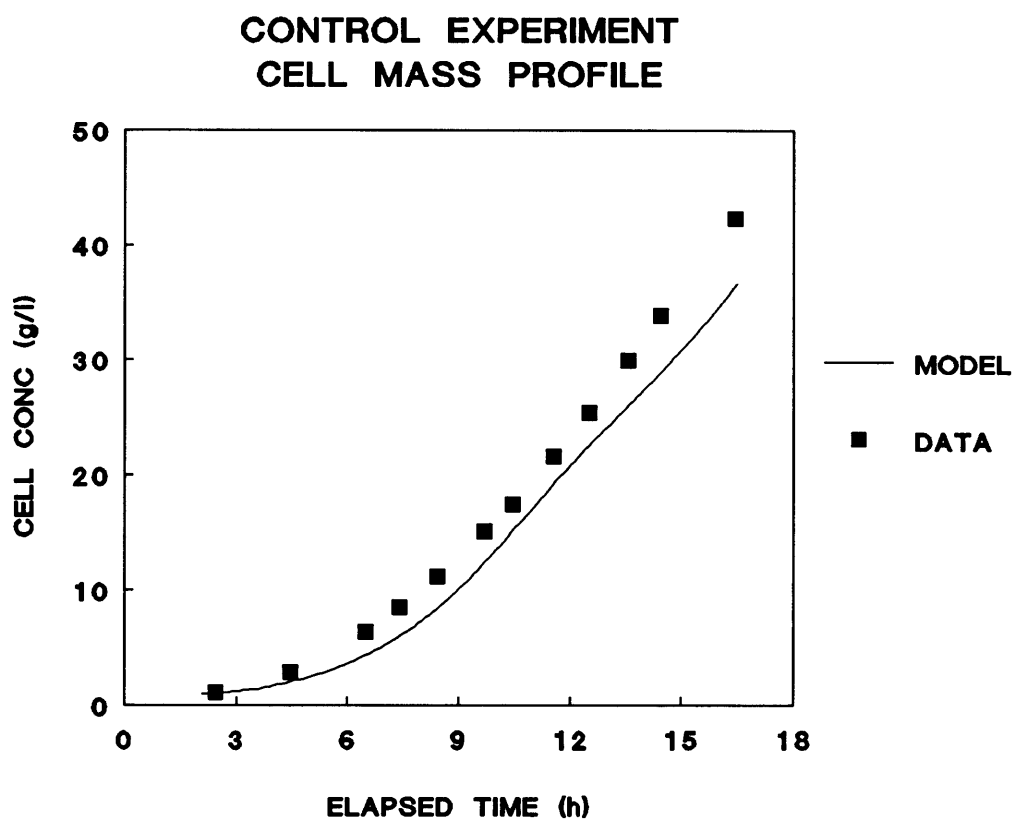


Figure E37: Cell concentration profile during the control fermentation.

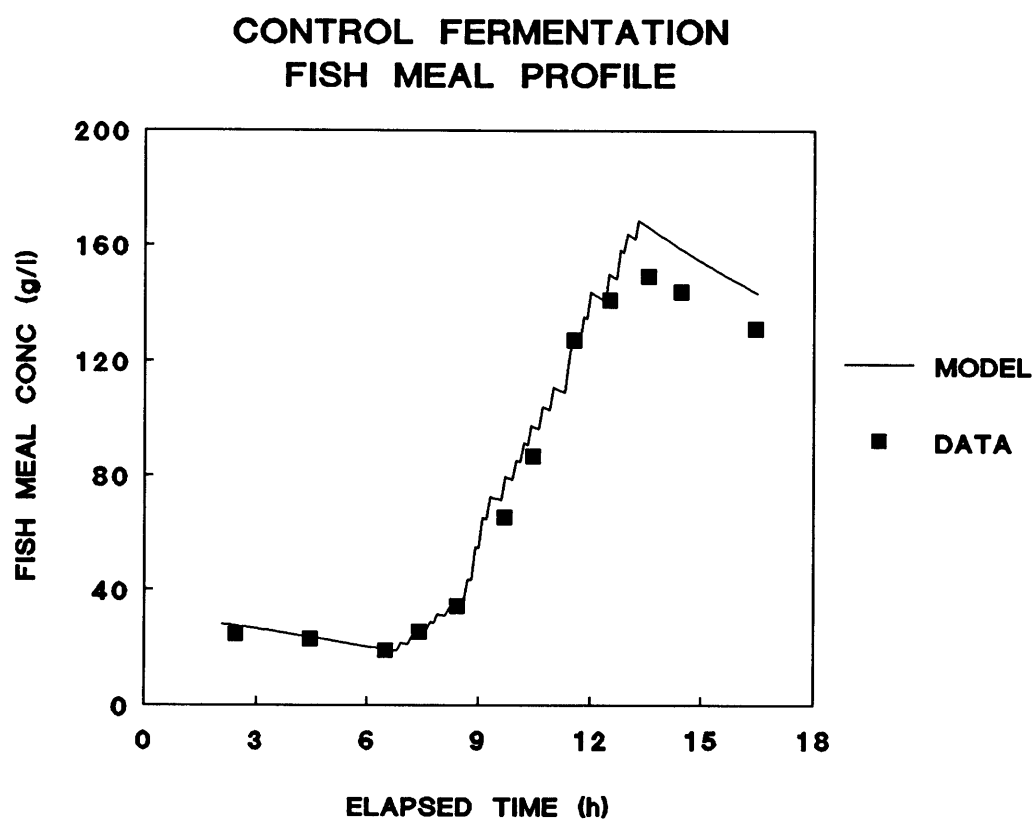


Figure E38: The fishmeal profile during the control fermentation.

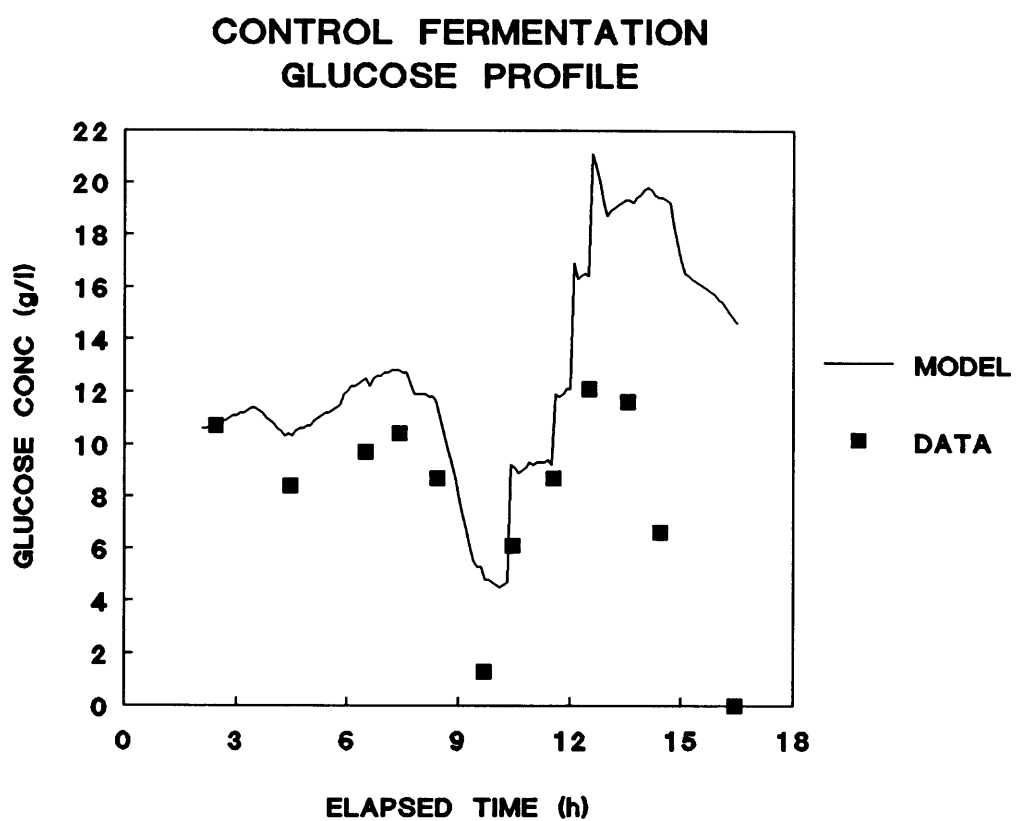


Figure E39: The glucose profile during the control fermentation.

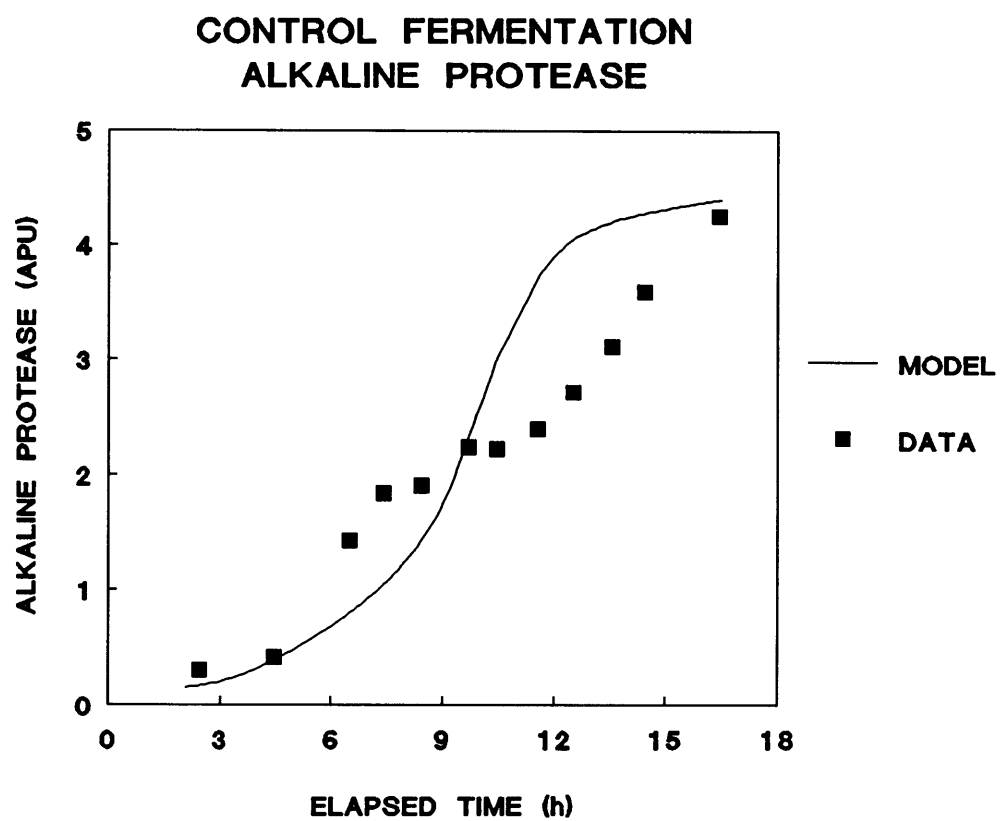


Figure E40: The alkaline protease activity profile during the control fermentation.

E.11) Summary of the Model

<u>MATERIAL</u>	<u>EQUATION</u>
CELLS	$\frac{dX}{dt} = \mu_{MAX} \cdot \frac{D}{K_D + D} \cdot X$
GLUCOSE	$-\frac{dG}{dt} = -\frac{dG_A}{dt} + \frac{1}{K_{\frac{X}{G}}} \cdot \frac{dX}{dt}$
GLUCOSE CONSUMPTION	$K_{\frac{X}{G}} = K_{\alpha} + \frac{K_{\beta} \cdot D}{K_{\gamma} + D}$
DISSOLVED FISHMEAL	$-\frac{dD}{dt} = \frac{dF}{dt} + \frac{1}{Y_{\frac{X}{D}}} \cdot \frac{dX}{dt}$
FISHMEAL	$-\frac{dF}{dt} = -\frac{dF_A}{dt} + \frac{F^{1.9} \cdot \psi \cdot \gamma_{MAX} \cdot N}{K_F + N}$
NEUTRAL PROTEASE	$\frac{dN}{dt} = \alpha_N \mu X \exp(-K_{ND}D) \exp(-K_{NG}G)$
ALKALINE PROTEASE	$\frac{dA}{dt} = \alpha_A \mu X \exp(-K_{AD}D) \exp(-K_{AG}G)$
TOXIC PRODUCT	$\mu = (T_1 - T_2 A_E) \cdot (T_1 - T_3 A_N)$

<u>PARAMETER</u>	<u>FITTED VALUE</u>	<u>INDEPENDENTLY DETERMINED VALUE</u>
μ_{MAX}		0.91 1/h
K_D	3.9 g/l	
$Y_{X/D}$		0.92 g/g
K_α	0.30 g/g	
K_β	0.18 g/g	
K_γ	1.29 g/l	
$\psi \cdot \gamma_{\text{MAX}}$	$0.00968 \text{ l}^{0.9}/\text{g}^{0.9}\text{-h}$	
K_F	0.32 NPU	
α_N	0.543 NPU/l-g	
α_A	0.556 APU/l-g	
K_{ND}	0.131 1/g	
K_{NG}	0.050 1/g	
K_{AD}	0.104 1/g	
K_{AG}	0.056 1/g	
T_1	0.670 1/h	
T_2	$0.048 \text{ l/g-h}^{0.5}$	
T_3	$0.034 \text{ l/g-h}^{0.5}$	

E.12) The Limitations to Alkaline Protease Production

The model and the control fermentation were useful in determining the factors that limit the production of alkaline protease. The hierarchy of limitations representing the series of problems encountered in the development of the process are as follows:

- 1) Regulation of protease production by glucose and amino acids.
- 2) Oxygen transfer limitation.
- 3) Toxic end product accumulation.
- 4) Filling up of the reactor.
- 5) Physical limitations, for example, viscosity and foaming.

E.13) Strengths and Areas for Further Improvement.

The strengths of the model are as follows:

- 1) Shows the importance of regulation in controlling protease production.
- 2) Identified the toxic end product effect.
- 3) Was used to develop a hierarchy of limitations.
- 4) Shows the risks of model extrapolation.
- 5) Helps a reasoned solid substrate feeding strategy to be determined.

The areas for further improvement are as follows:

- 1) All the amino acids are lumped as dissolved fishmeal.
- 2) There is no independent measurement of the dissolved fishmeal to validate the

model.

- 3) A Monod expression is assumed to but this expression may not apply under non-steady state conditions.
- 4) Product formation is assumed proportional to growth. This assumption does not consider protease formation in the stationary phase.
- 5) The glucose and fishmeal concentrations are determined using a mas balance. The problem with this approach is that the concentrations supplied and consumed are much greater than the aqueous concentrations. Thus the aqueous concentration is a small difference between two large numbers. Such a number has a large degree of error.
- 6) The model has 15 fitted parameters. This large number should be reduced or more parameters independently verified.
- 7) Initial conditions at the beginning of the fermentation are needed but these are not immediately available during the fermentation.
- 8) Data is available for only a limited range of conditions, which means that extrapolation of the data will be necessary for a description of all the possible variable values.
- 9) There is no description of acetate and acetoin production and hence strategies for avoiding their formation or toxic effects cannot be developed.

APPENDIX F

BASIC Program Used to Generate Model

Fermentation Profiles

```

10 'PROGRAM NAME BA.BAS
20 'START DATE: 14 DECEMBER, 1989
30 'WRITTEN BY MAX J. KENNEDY
40 '
50 'THIS PROGRAM IS THE LATEST VERSION OF THE SOLID SUBSTRATE
60 'FERMENTATION MODEL. THIS NEW VERSION TAKES INTO CONSIDERATION
70 'ALL 4 FERMENTATIONS AND ALSO THE REPRESSIVE EFFECT OF GLUCOSE.
80 'ALSO INCLUDED ARE BALANCES ON GLUCOSE AND A GLUCOSE LIMITED
90 'GROWTH EXPRESSION.
100 '
110 '
120 'NOTE THAT THE GLUCOSE AND FISH MEAL FEED PROFILES CONTAIN THE
130 'AMOUNT ADDED AT A SPECIFIC TIME
140 '
150 '
160 ' AMENDED 3/19/90 TO TAKE INTO CONSIDERATION A TOXIC PRODUCT
170 ' AMENDED 3/20/90 WITH NEW VALUES FOR THE REPRESSION PARAMETERS
180 ' WHICH ARE BASED ON DATA FROM 5 FERMENTATIONS
190 ' AMENDED 3/23/90 TO CONSIDER BOTH 2,3-BUTANDIOL AND
200 ' ACETATE AS TOXIC PRODUCTS
210 ' AMENDED 4/22/90 TO CORRECTLY CONSIDER ACETATE AND ACETOIN
220 ' AS THE IHIIBITORS OF GROWTH. THE CONCENTRATIONS OF ACETATE
230 ' AND ACETOIN WERE DETERMINED BY THE HPLC
240 '
250 '
260 'OPENING OF INPUT AND OUTPUT FILES
270 '
280 OPEN "MOD1.OUT" FOR OUTPUT AS #1
290 OPEN "MOD2.OUT" FOR OUTPUT AS #4
300 A=3
310 GOSUB 3910
320 LINE INPUT "What fermentation number is to be simulated ? "; F$
330 B=1
340 QGLU$="GLU"+F$+".INP"
350 QFM$ = "FISH"+F$+".INP"
360 QTOX$="TOX"+F$+".INP"
370 QACET$="ACET"+F$+".INP"
380 OPEN QGLU$ FOR INPUT AS #2
390 OPEN QFM$ FOR INPUT AS #3
400 OPEN QTOX$ FOR INPUT AS #5
410 OPEN QACET$ FOR INPUT AS #6

```

```
420 '  
430 'DIMENSION STATEMENTS  
440 '  
450 DIM TIMG(700)  
460 DIM AMTG(700)  
470 DIM TIMF(700)  
480 DIM AMTF(700)  
490 DIM FSUB(700)  
500 DIM DFSUB(700)  
510 DIM TIMT(700)  
520 DIM AMTT(700)  
530 DIM TIMA(700)  
540 DIM AMTA(700)  
550 '  
560 'EQUATION PARAMETERS  
570 '  
580 '  
590 ' PARAMETERS TO DESCRIBE GROWTH  
600 '  
610 ZMUMAX = .91  
620 ZMUMA = .91  
630 ZKD = 3.9  
640 ZKG = 4.1  
650 '  
660 ' YIELD COEFFIECENTS  
670 '  
680 YXD = .92  
690 YXG = .347  
700 '  
710 ' PARAMETERS TO DESCRIBE FISHMEAL DEGRADATION  
720 '  
730 DEGMAX = .00968  
740 DEGN = .32  
750 '  
760 ' PARAMETERS USED TO DESCRIBE REPRESSION  
770 '  
780 ALPHAN = .544  
790 ALPHA A = .556  
800 DELND = -.131  
810 DELNG = -.05  
820 DELAD = -.104  
830 DELAG = -.056  
840 '  
850 ' PARAMETERS TO DESCRIBE TOXIC PRODUCT EFFECT  
860 '  
870 ACETA=.67  
880 ACETB=-.034  
890 ACETC=-.048  
900 '  
910 'PARAMETERS TO DETERMINE TOTAL ELAPSED TIME  
920 '
```

```

930 TOTAL = 15
940 IF F$="13" THEN TOTAL = 13
950 IF F$="16" THEN TOTAL = 16.5
960 Z = .1
970 '
980 'INPUT OF THE INITIAL CONDITIONS
990 '
1000 A =2
1010 GOSUB 3910
1020 INPUT "What is the initial cell conc, Xo (g/l) ";XO
1030 GOSUB 3910
1040 INPUT "What is the initial dissolved fish meal conc, Do (g/l) ";DO
1050 GOSUB 3910
1060 INPUT "What is the initial neutral protease conc, No (NPU) ";ANO
1070 GOSUB 3910
1080 INPUT "What is the initial alkaline protease conc, Ao (APU) ";AO
1090 GOSUB 3910
1100 INPUT "What is the length of the lag phase, (h) ";TINIT
1110 GOSUB 3910
1120 '
1130 'INPUT OF THE GLUCOSE FEED PROFILE
1140 '
1150 INPUT #2, NN
1160 FOR XX=1 TO NN
1170 INPUT #2, TIMG(XX)
1180 NEXT XX
1190 FOR XXX=1 TO NN
1200 INPUT #2, AMTG(XXX)
1210 NEXT XXX
1220 '
1230 'INPUT OF THE FISH MEAL FEED PROFILE
1240 '
1250 INPUT #3, NNN
1260 FOR PP=1 TO NNN
1270 INPUT #3, TIMF(PP)
1280 NEXT PP
1290 FOR QQ=1 TO NNN
1300 INPUT #3, AMTF(QQ)
1310 NEXT QQ
1320 '
1330 ' INPUT OF THE ACETOIN CONCENTRATIONS
1340 '
1350 INPUT #5, NM
1360 FOR JJ= 1 TO NM
1370 INPUT #5, TIMT(JJ)
1380 NEXT JJ
1390 FOR JJJ= 1 TO NM
1400 INPUT #5, AMTT(JJJ)
1410 NEXT JJJ
1420 '
1430 ' INPUT OF THE ACETATE CONCENTRATION

```

```

1440 '
1450 INPUT #6, AC
1460 FOR JK = 1 TO AC
1470 INPUT #6, TIMA(JK)
1480 NEXT JK
1490 FOR JFK=1 TO AC
1500 INPUT #6, AMTA(JFK)
1510 NEXT JFK
1520 '
1530 'PRINTING OF THE INITIAL CONDITIONS, PARAMETERS AND FEED PROFILES
1540 '
1550 PRINT #1, USING "FERMENTATION \\ SIMULATION";F$
1560 B=4
1570 GOSUB 3990
1580 PRINT #1,"INITIAL CONDITIONS"
1590 B=2
1600 GOSUB 3990
1610 PRINT #1, USING "CELL CONC          ### g/l";XO
1620 PRINT #1, USING "FISH MEAL CONC      ### g/l";AMTF(1)
1630 PRINT #1, USING "GLUCOSE CONC        ### g/l";AMTG(1)
1640 PRINT #1, USING "DISSOLVED FISH MEAL CONC  ### g/l";DO
1650 PRINT #1, USING "NEUTRAL PROTEASE CONC  ### NPU";ANO
1660 PRINT #1, USING "ALKALINE PROTEASE CONC  ### APU";AO
1670 PRINT #1, USING "END OF LAG PHASE      ### h ";TINIT
1680 B=4
1690 GOSUB 3990
1700 PRINT #1, "EQUATION PARAMETERS"
1710 B=2
1720 GOSUB 3990
1730 PRINT #1, USING "MUMAX    = ### 1/h";ZMUMAX
1740 PRINT #1, USING "Kd      = ### g/l";ZKD
1750 PRINT #1, USING "Kg      = ### g/l";ZKG
1760 PRINT #1, USING "Yx/d    = ### g/g";YXD
1770 PRINT #1, USING "Yx/g    = ### g/g";YXG
1780 PRINT #1, USING "DEGMAX   = ##### ";DEGMAX
1790 PRINT #1, USING "Kn       = ### NPU";DEGN
1800 PRINT #1, USING "ALPHA N  = ### NPU/g";ALPHAN
1810 PRINT #1, USING "ALPHA A  = ### APU/g";ALPHAA
1820 PRINT #1, USING "DELND    = ### l/g";DELND
1830 PRINT #1, USING "DELNG    = ### l/g";DELNG
1840 PRINT #1, USING "DELAD    = ### l/g";DELAD
1850 PRINT #1, USING "DELAG    = ### l/g";DELAG
1860 PRINT #1, USING "STEP SIZE = ##### h";Z
1870 PRINT #1, USING "FERM TIME = ### h";TOTAL
1880 PRINT #1, USING "ACETA    = ### 1/h";ACETA
1890 PRINT #1, USING "ACETB    = ### l/g";ACETB
1900 PRINT #1, USING "ACETC    = ### l/g";ACETC
1910 B=4
1920 GOSUB 3990
1930 PRINT #1, "FISH MEAL FEED PROFILE"
1940 B=1

```

```

1950 GOSUB 3990
1960 PRINT #1, "TIME OF FEED      CONC OF FISH MEAL FED"
1970 PRINT #1, "  (h)              (g/l)"
1980 PRINT #1, " "
1990 FOR X=1 TO NNN
2000 PRINT #1, USING "  ###.###          ###.### ";TIMF(X),AMTF(X)
2010 NEXT X
2020 B=5
2030 GOSUB 3990
2040 PRINT #1, "ACETOIN CONCENTRATION PROFILE"
2050 PRINT #1, " "
2060 PRINT #1, "  TIME          ACETOIN CONC"
2070 PRINT #1, "  (h)              (g/l)"
2080 PRINT #1, " "
2090 FOR VJ = 1 TO NM
2100 PRINT #1, USING "  ###.###          ###.### ";TIMT(VJ),AMTT(VJ)
2110 NEXT VJ
2120 GOSUB 3990
2130 PRINT #1, "ACETATE FEED PROFILE"
2140 PRINT #1, " "
2150 PRINT #1, "  TIME          ACETATE CONC"
2160 PRINT #1, "  (h)              (g/l)"
2170 PRINT #1, " "
2180 FOR VJA=1 TO AC
2190 PRINT #1, USING "  ###.###          ###.### ";TIMA(VJA),AMTA(VJA)
2200 NEXT VJA
2210 GOSUB 3990
2220 PRINT #1, "GLUCOSE FEED PROFILE"
2230 PRINT #1, " "
2240 PRINT #1, "  TIME OF FEED      CONC GLUC ADDED"
2250 PRINT #1, "  (h)              (g/l)"
2260 PRINT #1, " "
2270 FOR RR=1 TO NN
2280 PRINT #1, USING "  ###.###          ###.### ";TIMG(RR),AMTG(RR)
2290 NEXT RR
2300 PRINT #4, USING "FERMENTATION \\ SIMULATION";F$
2310 C=5
2320 GOSUB 4060
2330 PRINT #4, " TIME  X    MU    F    D    G  ALK  NEUT  LIMITATION"
2340 PRINT " TIME X    MU    F    D    G  ALK  NEUT  YXG  T  A  LIMIT"
2350 C=2
2360 GOSUB 4060
2370 PRINT " "
2380 '
2390 'SETTING VARIABLES TO INITIAL CONDITIONS
2400 '
2410 D=DO
2420 X=XO
2430 A=AO
2440 AN=ANO
2450 G=AMTG(1)

```

```

2460 F=AMTF(1)
2470 FF=AMTF(1)
2480 T=0!
2490 ZAC=0!
2500 TIME=TINIT
2510 '
2520 ' START OF EACH TIME STEP
2530 '
2540 NUMBR=(TOTAL-TINIT)/Z
2550 J=2
2560 FSUB(1)=AMTF(1)
2570 FOR M=1 TO NUMBR
2580 '
2590 'CALCULATION OF GROWTH RATE AND GROWTH LIMITATION
2600 '
2610 ZMUD = ZMUMAX*D/(D+ZKD)
2620 ZMUG = ZMUMA*G/(G+ZKG)
2630 IF ZMUD < ZMUG THEN 2640 ELSE 2670
2640 ZMU = ZMUD
2650 S$ = "FISHMEAL"
2660 GOTO 2690
2670 ZMU = ZMUG
2680 S$ = "GLUCOSE"
2690 ZMUT=ACETA*ACETA+ACETA*ACETB*T+ACETA*ACETC*ZAC+ACETB*ACETC*ZAC*T
2700 IF T=0! AND ZAC=0! THEN ZMUT=99
2710 IF ZMUT<ZMU THEN 2720 ELSE 2740
2720 ZMU=ZMUT
2730 S$="ENDPROD"
2740 '
2750 'BALANCE ON GLUCOSE
2760 '
2770 YXG=.3+.18*D/(1.29+D)
2780 GCON=-1/YXG*DXDT
2790 ADDSTEP=0!
2800 FOR V=2 TO NN
2810 IF TIMG(V) >= TIME AND TIMG(V) < TIME+Z THEN ADDSTEP=ADDSTEP+AMTG(V)
2820 NEXT V
2830 GADD=ADDSTEP/Z
2840 '
2850 'BALANCE ON FISH MEAL
2860 '
2870 FADD=0!
2880 FOR W=2 TO NNN
2890 IF TIMF(W) >= TIME AND TIMF(W) < TIME+Z THEN FADD=FADD+AMTF(W)
2900 NEXT W
2910 IF ABS(FADD) > 0 THEN 2920 ELSE 2940
2920 FSUB(J)=FADD
2930 J=J+1
2940 FF=0!
2950 DFDTD=0!
2960 FOR Y=1 TO NNN

```

```

2970 DFSUB(Y)=-1*(FSUB(Y)^1.9)*DEGMAX*AN/(AN+DEGN)
2980 FSUB(Y)=FSUB(Y)+DFSUB(Y)*Z
2990 IF FSUB(Y) < 0! THEN FSUB(Y)=0!
3000 DFDTD=DFDTD+DFSUB(Y)
3010 FF=FF+FSUB(Y)
3020 NEXT Y
3030 '
3040 'BALANCE ON THE DISSOLVED FISH MEAL
3050 '
3060 DDDTD=-1*(DFDT+(1/YXD)*DXDT)
3070 '
3080 '
3090 'BALANCE ON NEUTRAL PROTEASE
3100 '
3110 DNDTD=ALPHAN*ZMU*X*EXP(DELND*D)*EXP(DELNG*G)
3120 '
3130 'BALANCE ON ALKALINE PROTEASE
3140 '
3150 DADTD=ALPHAA*ZMU*X*EXP(DELAD*D)*EXP(DELAG*G)
3160 '
3170 'CALCULATION OF THE DERIVATIVES
3180 '
3190 DXDT=ZMU*X
3200 DGDТ=GCON+GADD
3210 DFDT=DFDTD
3220 DDDT=DDDTD
3230 DNDT=DNDTD
3240 DADT=DADTD
3250 '
3260 'PREDICTING VALUES AT NEXT TIME STEP
3270 'USING THE EULER METHOD
3280 '
3290 X=X+DXDT*Z
3300 AN=AN+DNDT*Z
3310 A=A+DADT*Z
3320 D=D+DDDT*Z
3330 G=G+DGDТ*Z
3340 F=F+DFDT*Z+FADD
3350 '
3360 ' SETTING THE ACETOIN CONCENTRATION
3370 '
3380 FOR VY = 2 TO NM
3390 IF TIMT(VY) >= TIME AND TIMT(VY) < TIME+Z THEN T=AMTT(VY)
3400 NEXT VY
3410 '
3420 ' SETTING THE ACETATE CONCENTRATION
3430 '
3440 FOR VYA = 1 TO AC
3450 IF TИA(VYA) >= TIME AND TИA(VYA) < TIME+Z THEN ZAC=AMTA(VYA)
3460 NEXT VYA
3470 '

```

```

3480 'PREVENTION OF IMPOSSIBLE NEGATIVE CONCENTRATIONS
3490 '
3500 IF X<0 THEN X=0
3510 IF AN<0 THEN AN=0
3520 IF A<0 THEN A=0
3530 IF D<0 THEN D=0
3540 IF G<0 THEN G=0
3550 IF F<0 THEN F=0
3560 '
3570 'WATCHING FOR NUTRIENT EXHAUSTION
3580 '
3590 IF D=0 THEN 3600 ELSE 3610
3600 PRINT "WARNING: DISSOLVED FISH MEAL EXHAUSTED"
3610 IF G=0 THEN 3620 ELSE 3630
3620 PRINT "WARNING: GLUCOSE EXHAUSTED"
3630 IF F=0 THEN 3640 ELSE 3650
3640 PRINT "WARNING: FISH MEAL EXHAUSTED"
3650 '
3660 'WATCHING FOR INVALID VALUES OF SLOPES
3670 '
3680 IF DXDT<0 THEN 3690 ELSE 3710
3690 PRINT "ERROR: CELL DEATH OCCURRING"
3700 PRINT #4,"ERROR: CELL DEATH OCCURRING"
3710 IF DNDDT<0 THEN 3720 ELSE 3740
3720 PRINT "ERROR: NEUTRAL PROTEASE DESTRUCTION"
3730 PRINT #4, "ERROR: NEUTRAL PROTEASE DESTRUCTION"
3740 IF DADT<0 THEN 3750 ELSE 3770
3750 PRINT "ERROR: ALKALINE PROTEASE DESTRUCTION"
3760 PRINT #4, "ERROR: ALKALINE PROTEASE DESTRUCTION"
3770 IF DFDT>0 THEN 3780 ELSE 3800
3780 PRINT "ERROR: FISH MEAL BEING CREATED"
3790 PRINT #4, "ERROR: FISH MEAL BEING CREATED"
3800 '
3810 'INCREMENTING THE TIME STEP
3820 '
3830 TIME=TIME+Z
3840 '
3850 'PRINTING THE RESULTS TO THE SCREEN AND TO A FILE
3860 '
3870 PRINT USING "##.## ##.## ##.### ##.### ##.### ##.### ##.### ##.### ##.### ##.### ##.### ##.###"
&";TIME,X,ZMU,F,D,G,A,AN,YXG,T,ZAC,S$
3880 PRINT #4, USING "##.### ##.### ##.### ##.### ##.### ##.### ##.### ##.### ##.### ##.### ##.###"
&";TIME,X,ZMU,F,D,G,A,AN,S$
3890 NEXT M
3900 END
3910 '
3920 'SUBROUTINE TO PRINT A BLANK LINES TO THE SCREEN
3930 '
3940 FOR N=1 TO A
3950 PRINT " "
3960 NEXT N

```



```
3970 RETURN
3980 '
3990 'SUBROUTINE TO PRINT B BLANK LINES TO FILE #1
4000 '
4010 FOR SS=1 TO B
4020 PRINT #1, " "
4030 NEXT SS
4040 RETURN
4050 '
4060 'SUBROUTINE TO PRINT C BLANK LINES TO FILE #4
4070 '
4080 FOR TT = 1 TO C
4090 PRINT #4, " "
4100 NEXT TT
4110 RETURN
```

APPENDIX G

FORTTRAN Program Used to Optimize the

Solid Substrate Model

```
PROGRAM FISHMEAL
IMPLICIT REAL*8(A-H,O-Z)
```

```
C   COMPOSED OF FOLLOWING PROGRAM SEGMENTS.
C   1) FINAL TIME SETTING.
C   2) INITIAL GUESS OF CONTROL(OPEN LOOP FISHMEAL FEED RATE) PROFILE.
C   3) STATE FORWARD EVOLUTION.
C   4) COSTATE BACKWARD EVOLUTION.
C     4.A) COSTATE BACKWARD.
C     4.B) UPDATE OF CONTROL PROFILE.
C     4.C) BREAK OF SINGULAR ARC.
C     4.D) LOCATION OF DEVIATION POINT BY INITIAL STATE.
C   5) GOTO 3) AND REPEAT 3) AND 4).
C
```

```
REAL*8 COS1(500),COS2(500),COS3(500),COS4(500),COS5(500)
COMMON /BK0/ QFMIN,QFMAX
COMMON /BK1/ X(500),D(500),PN(500),PA(500),F(500)
COMMON /BK2/ CONTROL(500),PCONTROL(500)
COMMON /BK3/ IK
COMMON /BK4/ JK,CROSS
COMMON /BK5/ FISHML
COMMON /BK6/ DZERO(500)
```

```
C
REAL*8 FY(5),BY(5),C(24),W(5,9)
EXTERNAL FORW,BACKW,DUMJ
```

```
C   SEGMENT 1: INITIALIZATION -----
C
```

```
QFMIN = 0.0
QFMAX = 2000.0
```

```
WRITE(*,*) 'ENTER THE TOTAL ITERATION NUMBER.'
READ(*,*) ISTOP
WRITE(*,*) 'ENTER THE FINAL TIME IN HOURS.'
READ(*,*) FHOURS
WRITE(*,*) 'ENTER THE TIME INCREMENT, DT.'
READ(*,*) DT
NFCOUNT = FHOURS/DT+1
WRITE(*,*) 'ENTER THE BETA-VALUE ( LOW VALUE IS CONSERVATIVE.)'
READ(*,*) BETA
```

```

C
C
C   CALL GUESS(DT,NFCOUNT,NBREAK)
C
C   THIS POINT IS THE BIGGEST LOOPING OF THE WHOLE PROGRAM.

```

```

    ITERA = -1
  100 ITERA = ITERA + 1

```

```

    X(1) = 1.0
    D(1) = 0.5
    PN(1) = 0.07
    PA(1) = 0.07
    F(1) = FISHML

```

```

C
  INDEX = 1
  WRITE(*,*) 'GETTING INTO FORWARD.'
  DO 300 IK=1,NFCOUNT-1,1
    T1DBL = (IK-1)*DT
    T2DBL = IK*DT
    FY(1) = X(IK)
    FY(2) = D(IK)
    FY(3) = PN(IK)
    FY(4) = PA(IK)
    FY(5) = F(IK)
    CALL DVERK(5,FORW,T1DBL,FY,T2DBL,1.0D-4,INDEX,C,5,W,IER)
    X(IK+1) = FY(1)
    D(IK+1) = FY(2)
    PN(IK+1) = FY(3)
    PA(IK+1) = FY(4)
    F(IK+1) = FY(5)
  300 CONTINUE

```

```

    COS1(NFCOUNT) = 0.0
    COS2(NFCOUNT) = 0.0
    COS3(NFCOUNT) = 0.0
    COS4(NFCOUNT) = -1.0
    COS5(NFCOUNT) = 0.0

```

```

C
  D(NFCOUNT) = 2.04874985094

```

```

C
  INDEX = 1
  CROSS = 0.0

```

```

C
  WRITE(*,*) 'GETTING INTO BACKWARD.'
  DO 500 JK = NFCOUNT,2,-1
    T1DBL = (NFCOUNT-JK)*DT
    T2DBL = (NFCOUNT+1-JK)*DT
    BY(1) = COS1(JK)
    BY(2) = COS2(JK)

```

```

BY(3) = COS3(JK)
BY(4) = COS4(JK)
BY(5) = COS5(JK)
CALL DVERK(5,BACKW,T1DBL,BY,T2DBL,1.0D-4,INDEX,C,5,W,IER)
COS1(JK-1) = BY(1)
COS2(JK-1) = BY(2)
COS3(JK-1) = BY(3)
COS4(JK-1) = BY(4)
COS5(JK-1) = BY(5)

```

```

CALL GETSING(COS1(JK-1),COS4(JK-1),DS)

```

```

DZP = DZERO(JK)
DZ = DZERO(JK-1)
DP = D(JK)

```

```

IF(CROSS.GT.0.5) THEN
  D(JK-1) = DZERO(JK-1)
  CONTROL(JK-1) = 0.0
  GOTO 450
ENDIF

```

```

IF(DP.GT.DZP.AND.DS.GT.DZ) THEN
  D(JK-1) = DS
  CALL SEARCHQ(DT,QVALUE)
  CONTROL(JK-1) = QVALUE
ENDIF

```

```

IF(DP.GT.DZP.AND.DS.LE.DZ) THEN
  D(JK-1) = DZERO(JK-1)
  CALL SEARCHQ(DT,QVALUE)
  CONTROL(JK-1) = QVALUE
  CROSS = 1.0
ENDIF

```

```

450 CONTINUE
500 CONTINUE

```

```

DO 600 L=1,NFCOUNT,1
  WRITE(*,*) L, CONTROL(L),D(L),F(L)
  PCONTROL(L) = (1.0-BETA)*PCONTROL(L) + BETA*CONTROL(L)
600 CONTINUE

```

```

IF(ITERA.LT.ISTOP) GOTO 100
OPEN(8,FILE='STATE.DAT',STATUS='NEW')
OPEN(9,FILE='COSTA.DAT',STATUS='NEW')
DO 800 M=1,NFCOUNT,1
  WRITE(8,911) M,CONTROL(M),X(M),D(M),PN(M),PA(M),F(M)
  WRITE(9,912) M,COS1(M),COS2(M),COS3(M),COS4(M),COS5(M)
800 CONTINUE

```

```

CLOSE(8)
CLOSE(9)
911 FORMAT(2X,I4,2X,6(D12.6,2X))
912 FORMAT(2X,I4,2X,5(D12.6,2X))
STOP
END

```

```

SUBROUTINE FORW(N,XTIME,Y,YD)
IMPLICIT REAL*8(A-H,O-Z)
INTEGER N
REAL*8 Y(N),YD(N),XTIME
COMMON /BK2/ CONTROL(500),PCONTROL(500)
COMMON /BK3/ IK
C
REAL MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
DATA MUMAX,KD,YXD,PHI,KF/0.91,3.9,0.92,0.00968,0.32/
DATA ALFA,ALFN,KND,KNG,KAD,KAG/2.54,5.76,-0.38,-0.32,-0.32,-0.23/
DATA KBIG,GLUC/8.0,8.7/
C
FMU(XD) = MUMAX*XD/(XD+KD)/(XD+KBIG)*KBIG
FFD(XFF,XPN) = PHI*XFF**1.9*XPN/(XPN+KF)
FMUMON(XD) = MUMAX*XD/(XD+KD)
FNP(XD) = ALFN*FMUMON(XD)*EXP(KND*XD)*EXP(KNG*GLUC)
FAP(XD) = ALFA*FMUMON(XD)*EXP(KAD*XD)*EXP(KAG*GLUC)
C
DFMU(XD) = MUMAX*KBIG*(KD*KBIG-XD**2)/(XD+KD)**2/(XD+KBIG)**2
DFFDN(XFF,XPN) = PHI*XFF**1.9*KF/(KF+XPN)**2
DFFDF(XFF,XPN) = 1.9*PHI*XFF**0.9*XPN/(XPN+KF)
DFMUMON(XD) = MUMAX*KD/(XD+KD)**2
DFNP(XD) = ALFN*EXP(KNG*GLUC)*EXP(KND*XD)/(XD+KD)*
#(KD/(XD+KD)+KND*XD)
DFAP(XD) = ALFA*EXP(KAG*GLUC)*EXP(KAD*XD)/(XD+KD)*
#(KD/(XD+KD)+KAD*XD)
C
CELL = Y(1)
DISV = Y(2)
PNEU = Y(3)
PALK = Y(4)
FISH = Y(5)
C
YD(1) = FMU(DISV)*CELL
YD(2) = FFD(FISH,PNEU) - 1.0/YXD*FMU(DISV)*CELL
YD(3) = FNP(DISV)*CELL
YD(4) = FAP(DISV)*CELL
YD(5) = PCONTROL(IK) - FFD(FISH,PNEU)
C
RETURN
END

```

```

SUBROUTINE BACKW(N,XTIME,Y,YD)
  IMPLICIT REAL*8(A-H,O-Z)
  INTEGER N
  REAL*8 Y(N),YD(N),XTIME
  COMMON /BK1 / X(500),D(500),PN(500),PA(500),F(500)
  COMMON /BK4 / JK,CROSS

REAL MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
DATA MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
DATA ALFA,ALFN,KND,KNG,KAD,KAG/2.54,5.76,-0.38,-0.32,-0.23 /
DATA KBIG,GLUC/8.0,8.7 /

FMU(XD) = MUMAX*XD/(XD+KD)/(XD+KBIG)*KBIG
FED(XFF,XPN) = PHI*XFF**1.9*XPN/(XPN+KF)
FMUMON(XD) = MUMAX*XD/(XD+KD)
FNP(XD) = ALFN*FMUMON(XD)*EXP(KND*XD)*EXP(KNG*GLUC)
FAP(XD) = ALFA*FMUMON(XD)*EXP(KAD*XD)*EXP(KAG*GLUC)

DPMU(XD) = MUMAX*KBIG*(KD*KBIG-XD**2)/(XD+KD)**2/(XD+KBIG)**2
DFFDN(XFF,XPN) = PHI*XFF**1.9*KF/(KF+XPN)**2
DFFDF(XFF,XPN) = 1.9*PHI*XFF**0.9*XPN/(XPN+KF)
DPMUMON(XD) = MUMAX*KD/(XD+KD)**2
DPMU(XD) = ALFN*EXP(KNG*GLUC)*EXP(KND*XD)/(XD+KD)*
#(KD/(XD+KD)+KND*XD)
DFAP(XD) = ALFA*EXP(KAG*GLUC)*EXP(KAD*XD)/(XD+KD)*
#(KD/(XD+KD)+KAD*XD)

CELL = X(JK)
DISV = D(JK)
PNEU = PN(JK)
PALK = PA(JK)
FISH = F(JK)

CO1 = Y(1)
CO2 = Y(2)
CO3 = Y(3)
CO4 = Y(4)
CO5 = Y(5)

IF(CROSS,LT,0.5) SINGULAR = 0.0
IF(CROSS,GT,0.5) SINGULAR = CO1*DFMU(DISV) + CO4*DFAP(DISV)
YD(1) = (CO1-1.0/YXD*CO2)*FMU(DISV) + CO3*FNP(DISV) + CO4*FAP(DISV)
YD(2) = SINGULAR*CELL-1.0/YXD*CO2*DFMU(DISV)*CELL
# + CO3*DFNP(DISV)*CELL
YD(3) = (CO2-CO5)*DFFDN(FISH,PNEU)
YD(4) = 0.0
YD(5) = (CO2-CO5)*DFFDF(FISH,PNEU)

```



```

F(IK+1) = FY(5)
DZERO(IK+1) = FY(2)

```

300 CONTINUE

```

RETURN
END

```

```

SUBROUTINE FGUSS(N,XTIME,Y,YD)
IMPLICIT REAL*8(A-H,O-Z)
INTEGER N
REAL*8 Y(N),YD(N),XTIME
COMMON /SBK1/ QACTION

```

```

REAL*8 MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
DATA MUMAX,KD,YXD,PHI,KF/0.91,3.9,0.92,0.00968,0.32/
DATA ALFA,ALFN,KND,KNG,KAD,KAG/2.54,5.76,-0.38,-0.32,-0.23/
DATA KBIG,GLUC/8.0,8.7/

```

```

FMU(XD) = MUMAX*XD/(XD+KD)/(XD+KBIG)*KBIG
FFD(XFF,XPN) = PHI*XFF**1.9*XPN/(XPN+KF)
FMUMON(XD) = MUMAX*XD/(XD+KD)
FNP(XD) = ALFN*FMUMON(XD)*DEXP(KND*XD)*DEXP(KNG*GLUC)
FAP(XD) = ALFA*FMUMON(XD)*DEXP(KAD*XD)*DEXP(KAG*GLUC)

```

```

DFMU(XD) = MUMAX*KBIG*(KD*KBIG-XD**2)/(XD+KD)**2/(XD+KBIG)**2
DFFDN(XFF,XPN) = PHI*XFF**1.9*KF/(KF+XPN)**2
DFD(XFF,XPN) = 1.9*PHI*XFF**0.9*XPN/(XPN+KF)
DFMUMON(XD) = MUMAX*KD/(XD+KD)**2

```

```

DFNP(XD) = ALFN*DEXP(KNG*GLUC)*DEXP(KND*XD)/(XD+KD)*
#(KD/(XD+KD)+KND*XD)
DFAP(XD) = ALFA*DEXP(KAG*GLUC)*DEXP(KAD*XD)/(XD+KD)*
#(KD/(XD+KD)+KAD*XD)

```

```

CELL = Y(1)
DISV = Y(2)
PNEU = Y(3)
PALK = Y(4)
FISH = Y(5)

```

```

YD(1) = FMU(DISV)*CELL
YD(2) = FFD(FISH,PNEU) - 1.0/YXD*FMU(DISV)*CELL
YD(3) = FNP(DISV)*CELL
YD(4) = FAP(DISV)*CELL
YD(5) = QACTION - FFD(FISH,PNEU)

```

```

RETURN
END

```

SUBROUTINE DUMJ(N,X,Y,PD)


```

      IMPLICIT REAL*8(A-H,O-Z)
      INTEGER N
      REAL*8 X,Y(N),PD(N,N)
      RETURN
      END
C
C
      SUBROUTINE GETSING(CO1,CO4,DSING)
      IMPLICIT REAL*8(A-H,O-Z)
C
C   THIS SUBROUTINE TAKES THE TWO WEIGHTING FACTORS, CO1 AND CO4, AND
C   CALCULATE THE SOLUTION OF THE SINGULAR D-VALUE. THE PROBLEM, THOUGH,
C   IS THAT, DURING CERTAIN TIME PERIOD, THERE COULD BE MULTIPLE PROFLIES.
C   IN THIS CASE, THE TRUE SINGULAR VALUE IS JUDGED BY THE HAMILTONIAN.
C
      REAL MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
      DATA MUMAX,KD,YXD,PHI,KF/0.91,3.9,0.92,0.00968,0.32/
      DATA ALFA,ALFN,KND,KNG,KAD,KAG/2.54,5.76,-0.38,-0.32,-0.32,-0.23/
      DATA KBIG,GLUC/8.0,8.7/
C
      FMU(XD) = MUMAX*XD/(XD+KD)/(XD+KBIG)*KBIG
      FFD(XFF,XPN) = PHI*XFF**1.9*XPN/(XPN+KF)
      FMUMON(XD) = MUMAX*XD/(XD+KD)
      FNP(XD) = ALFN*FMUMON(XD)*EXP(KND*XD)*EXP(KNG*GLUC)
      FAP(XD) = ALFA*FMUMON(XD)*EXP(KAD*XD)*EXP(KAG*GLUC)
C
      DFMU(XD) = MUMAX*KBIG*(KD*KBIG-XD**2)/(XD+KD)**2/(XD+KBIG)**2
      DFFDN(XFF,XPN) = PHI*XFF**1.9*KF/(KF+XPN)**2
      DFFDF(XFF,XPN) = 1.9*PHI*XFF**0.9*XPN/(XPN+KF)
      DFMUMON(XD) = MUMAX*KD/(XD+KD)**2
      DFN(XD) = ALFN*EXP(KNG*GLUC)*EXP(KND*XD)/(XD+KD)*
      # (KD/(XD+KD)+KND*XD)
      DFAP(XD) = ALFA*EXP(KAG*GLUC)*EXP(KAD*XD)/(XD+KD)*
      # (KD/(XD+KD)+KAD*XD)
C
      FSING(XD) = -CO1*DFMU(XD)-CO4*DFAP(XD)
C
C
      TOL = ABS((CO1-0.001)*1.0E-7)
C
      DO 100 I=1,50,1
      DLOW = 0.5 + 6.0/50.0*FLOAT(I-1)
      DHIGH = 0.5 + 6.0/50.0*FLOAT(I)
      IF(DLOW.GT.6.0) THEN
          WRITE(*,*) '*** IN GETSING, CANNOT CROSS. ***'
          STOP
      ENDIF
      IF(ABS(FSING(DLOW)).LT.TOL) THEN
          DSING = DLOW
          RETURN
      ENDIF

```

```

      IF(ABS(FSING(DHIGH)).LT.TOL) THEN
          DSING = DHIGH
          RETURN
      ENDIF
      IF((FSING(DLOW).GT.0.0).AND.(FSING(DHIGH).LT.0.0)) GOTO 200
100 CONTINUE
C
C
200 CONTINUE
    DTOP = DHIGH
    DBOT = DLOW
300 CONTINUE
    D = (DTOP+DBOT)/2.0
    FVALUE = FSING(D)
    IF(ABS(FVALUE).LT.TOL) THEN
        DSING = D
        RETURN
    ENDIF
    IF(FVALUE.GT.0.0) THEN
        DBOT = D
        GOTO 300
    ENDIF
    IF(FVALUE.LT.0.0) THEN
        DTOP = D
        GOTO 300
    ENDIF
C
END

C
C
SUBROUTINE SEARCHQ(DT,Q)
IMPLICIT REAL*8(A-H,O-Z)
C
COMMON /BK1/ X(500),D(500),PN(500),PA(500),F(500)
COMMON /BK4/ JK,CROSS
C
REAL MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
DATA MUMAX,KD,YXD,PHI,KF/0.91,3.9,0.92,0.00968,0.32/
DATA ALFA,ALFN,KND,KNG,KAD,KAG/2.54,5.76,-0.38,-0.32,-0.32,-0.23/
DATA KBIG,GLUC/8.0,8.7/
C
FMU(XD) = MUMAX*XD/(XD+KD)/(XD+KBIG)*KBIG
FFD(XFF,XPN) = PHI*XFF**1.9*XPN/(XPN+KF)
FMUMON(XD) = MUMAX*XD/(XD+KD)
FNP(XD) = ALFN*FMUMON(XD)*EXP(KND*XD)*EXP(KNG*GLUC)
FAP(XD) = ALFA*FMUMON(XD)*EXP(KAD*XD)*EXP(KAG*GLUC)
C
DAVE = (D(JK-1)+D(JK))/2.0
CAVE = (X(JK-1)+X(JK))/2.0
Q = (F(JK)-F(JK-1))/DT + (D(JK)-D(JK-1))/DT + 1.0/YXD*FMU(DAVE)*CAVE

```

RETURN
END

APPENDIX H

FORTRAN Program Used to Determine the Fishmeal

Feed Profile After 12 Hours in the Confirmation Experiment

```

PROGRAM EXTEND
C
C THIS PROGRAM CALCULATES THE FISHMEAL FEED FOR THE PERIOD
C AFTER THE 12 HOUR OPTIMIZATION
C
C DEFINING THE VARIABLES
C
IMPLICIT REAL*8(A-H,O-Z)
REAL*8 FY(5),BY(5),C(24),W(5,9)
REAL MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
REAL*8 X(500),D(500),PN(500),PA(500),F(500)
REAL*8 YD(500),CONTROL(500)
C
C DATA STATEMENTS
C
DATA MUMAX,KD,YXD,PHI,KF/0.91,3.9,0.92,0.00968,0.32/
DATA ALFA,ALFN,KND,KNG,KAD,KAG/2.54,5.76,-0.38,-0.32,-0.32,-0.23/
DATA KBIG,GLUC/8.0,8.7/
C
C FUNCTION DEFINITIONS
C
FMU(XD) = MUMAX*XD/(XD+KD)/(XD+KBIG)*KBIG
FFD(XFF,XPN) = PHI*XFF**1.9*XPN/(XPN+KF)
FMUMON(XD) = MUMAX*XD/(XD+KD)
FNP(XD) = ALFN*FMUMON(XD)*EXP(KND*XD)*EXP(KNG*GLUC)
FAP(XD) = ALFA*FMUMON(XD)*EXP(KAD*XD)*EXP(KAG*GLUC)
C
C OPEN AN OUTPUT FILE
C
OPEN(UNIT=1,FILE='EXTEND.DAT')
WRITE(1,*) ' # Q X D PN PA F'
WRITE(1,*) ' '
C
C SEGMENT 1: INITIALIZATION -----
C
C WRITE(*,*) 'ENTER THE STARTING CONDITIONS.'
C WRITE(*,*) 'ENTER THE STARTING TIME IN HOUR : '
C READ(*,*) TIME1
C WRITE(*,*) 'ENTER THE FINISHING TIME IN HOUR : '
C READ(*,*) TIME2

```

```

C  WRITE(*,*) 'ENTER THE CELL DENSITY(X) : '
C  READ(*,*) XIN
C  WRITE(*,*) 'ENTER THE DISSOLVED FISHMEAL(D) : '
C  READ(*,*) DIN
C  WRITE(*,*) 'ENTER THE NEUTRAL PROTEASE(N) : '
C  READ(*,*) PNIN
C  WRITE(*,*) 'ENTER THE ALKALINE PROTEASE(A) : '
C  READ(*,*) PAIN
C  WRITE(*,*) 'ENTER THE FISHMEAL CONCENTRATION(F) : '
C  READ(*,*) FIN
C  WRITE(*,*) 'ENTER YOUR GUESS OF THE CONTROL ACTION(Q) : '
C  READ(*,*) Q
C
C  TIME1 = 12.0
C  TIME2 = 24.0
C  XIN = 28.4
C  DIN = 2.05
C  PNIN = 4.33
C  PAIN = 4.95
C  FIN = 35.1
C
C  ISTART = INT(1+TIME1/0.05)
C  IFINIS = INT(1+TIME2/0.05)
C
C  X(ISTART) = XIN
C  D(ISTART) = DIN
C  PN(ISTART) = PNIN
C  PA(ISTART) = PAIN
C  F(ISTART) = FIN
C
C  TIME = 12.0
C  DO 800 IK=ISTART,IFINIS,1
C
C  XTIME = 0.25/10.0
C  IF(IK.LE.ISTART+1) XTIME = 0.5/10.0
C  QHIGH = 550.0
C  QLOW = -10.0
C
C  200 CONTINUE
C  QTRY = (QHIGH+QLOW)/2.0
C
C  FY(1) = X(IK)
C  FY(2) = D(IK)
C  FY(3) = PN(IK)
C  FY(4) = PA(IK)
C  FY(5) = F(IK)
C
C  BREAKING DOWN INTO 4 TIME STEPS
C
C  DO 333 IM=1,10,1
C

```

C ADDING THE EULER METHOD AND REMOVING RUNGE-KUTTA METHOD

C

CELL = FY(1)

DISV = FY(2)

PNEU = FY(3)

PALK = FY(4)

FISH = FY(5)

C

YD(1) = FMUMON(DISV)*CELL

YD(2) = FFD(FISH,PNEU) - 1.0/YXD*FMUMON(DISV)*CELL

YD(3) = FNP(DISV)*CELL

YD(4) = FAP(DISV)*CELL

YD(5) = QTRY - FFD(FISH,PNEU)

C

C EULER SLOPE METHOD

C

FY(1)=FY(1)+XTIME*YD(1)

FY(2)=FY(2)+XTIME*YD(2)

FY(3)=FY(3)+XTIME*YD(3)

FY(4)=FY(4)+XTIME*YD(4)

FY(5)=FY(5)+XTIME*YD(5)

C

333 CONTINUE

C

C

C

BB=ABS(FY(2)-DIN)

WRITE(*,34)IK,BB,QTRY,FY(1)

34 FORMAT(3X,I4,3X,F7.4,3X,F5.1,3X,F5.1)

C

IF(ABS(FY(2)-DIN).LT.0.0001) THEN

CONTROL(IK) = QTRY

GOTO 500

ENDIF

C

IF(FY(2).GT.DIN) THEN

QHIG = QTRY

GOTO 200

ENDIF

C

IF(FY(2).LT.DIN) THEN

QLOW = QTRY

GOTO 200

ENDIF

C

C

500 CONTINUE

C

C UPDATING THE STATE PARAMETER VALUES

```
C      TIME = TIME + XTIME*10
      X(IK+1) = FY(1)
      D(IK+1) = FY(2)
      PN(IK+1) = FY(3)
      PA(IK+1) = FY(4)
      F(IK+1) = FY(5)
C
C      Q1=CONTROL(IK)
      WRITE(*,*) 'NEXT TIME STEP'
      WRITE(1,599)TIME,Q1,X(IK),D(IK),PN(IK),PA(IK),F(IK)
599  FORMAT(1X,F7.2,2X,F7.1,2X,F5.1,2X,F4.2,2X,F4.1,2X,F4.1,2X,F7.1)
      IF (FY(1).GT.200) THEN
          GOTO 900
      END IF
      800 CONTINUE
C
      900 CONTINUE
C
C      STOP
      END
```

APPENDIX I

FORTRAN Program Used to Estimate the PID Control

Parameters in the Dissolved Fishmeal Control Strategy

```

PROGRAM GAIN2
C
C THIS PROGRAM IS USED TO CALCULATE THE GAIN FACTOR FOR PI CONTROL
C
C DEFINING THE VARIABLES
C
IMPLICIT REAL*8(A-H,O-Z)
REAL*8 FY(5),PFY(5)
REAL MUMAX,KD,YXD,PHI,KF,ALFA,ALFN,KND,KNG,KAD,KAG,KBIG,GLUC
REAL*8 X(500),D(500),PN(500),PA(500),F(500)
REAL*8 YD(5),PYD(5),OFF2(500),DIFF(500)
REAL*8 PX(500),PD(500),PPN(500),PPA(500),PF(500)
REAL*8 QGAIN(500),ZMM(500),Q(500),XTIME(500)
C
C DATA STATEMENTS
C
DATA MUMAX,KD,YXD,PHI,KF/0.91,3.9,0.92,0.00968,0.32/
DATA ALFA,ALFN,KND,KNG,KAD,KAG/2.54,5.76,-0.38,-0.32,-0.32,-0.23/
DATA KBIG,GLUC,POWER/8.0,8.7,1.9/
C
C FUNCTION DEFINITIONS
C
FMU(XD) = MUMAX*XD/(XD+KD)/(XD+KBIG)*KBIG
FFD(XFF,XPN) = PHI*XFF**POWER*XPN/(XPN+KF)
FMUMON(XD) = MUMAX*XD/(XD+KD)
FNP(XD) = ALFN*FMUMON(XD)*EXP(KND*XD)*EXP(KNG*GLUC)
FAP(XD) = ALFA*FMUMON(XD)*EXP(KAD*XD)*EXP(KAG*GLUC)
C
C PERTURBATED MODEL FUNCTION DEFINITIONS
C
PFMU(XD) = PMUMAX*XD/(XD+PKD)/(XD+PKBIG)*PKBIG
PFFD(XFF,XPN) = PPHI*XFF**PPOWER*XPN/(XPN+PKF)
PFMUMON(XD) = PMUMAX*XD/(XD+PKD)
PFNP(XD) = PALFN*PFMUMON(XD)*EXP(PKND*XD)*EXP(PKNG*GLUC)
PFAP(XD) = PALFA*PFMUMON(XD)*EXP(PKAD*XD)*EXP(PKAG*GLUC)
C
C OPEN AN OUTPUT FILE
C
OPEN(UNIT=1,FILE='GAIN.DAT')
WRITE(1,*) 'CALCULATING THE GAIN'
WRITE(1,*) ' '

```



```

C
C  OPENING THE INPUT FILE
C
OPEN(UNIT=2,FILE='Q.PRN')
C
C  INPUT OF INITIALIZATION PARAMETERS
C
WRITE(*,*) 'THE PERTURBATION FACTORS'
WRITE(*,*) 'USUALLY BETWEEN 0.1-2.0'
WRITE(*,*) ' '
WRITE(*,*) 'ENTER THE CELL EQUATION FACTOR '
READ(*,*) ZPERTC
WRITE(*,*) 'ENTER THE DISSOLVED FISHMEAL EQUATION FACTOR '
READ(*,*) ZPERTD
WRITE(*,*) 'ENTER THE FISHMEAL EQUATION FACTOR '
READ(*,*) ZPERTF
WRITE(*,*) 'ENTER THE NEUTRAL PROTEASE EQUATION FACTOR '
READ(*,*) ZPERTN
WRITE(*,*) 'ENTER THE ALKALINE PROTEASE EQUATION FACTOR '
READ(*,*) ZPERTA
WRITE(*,*) ' '
WRITE(*,*) 'ENTER THE CONTROL PARAMETERS '
WRITE(*,*) 'PROPORTIONAL GAIN '
READ(*,*) GAMMA
WRITE(*,*) 'INTEGRAL GAIN '
READ(*,*) GAMMA2
WRITE(*,*) 'DIFFERENTIAL GAIN'
READ(*,*) G3
C
C  PERTURBING THE MODEL CONSTANTS
C
PMUMAX=ZPERTC*MUMAX
PKD=ZPERTC*KD
PYXD=ZPERTD*YXD
PPHI=ZPERTF*PHI
PKF=ZPERTF*KF
PALFA=ZPERTA*ALFA
PALFN=ZPERTN*ALFN
PKND=ZPERTN*KND
PKNG=ZPERTN*KNG
PKAD=ZPERTA*KAD
PKAG=ZPERTA*KAG
PKBIG=ZPERTC*KBIG
PPOWER=ZPERTF*POWER
C
C  READING IN THE CONTROL ACTION
C
DO 111 NN=1,206,1
READ(2,*) XTIME(NN),Q(NN)
C  WRITE(*,*) XTIME(NN),Q(NN)
111  CONTINUE

```

```

C
C  INITIALIZATION OF PARAMETERS
C
  OFFSET=0.0
  X(1)=10.1
  D(1)=3.32
  PN(1)=1.00
  PA(1)=1.23
  F(1)=15.6
  PX(1)=10.1
  PD(1)=3.32
  PPN(1)=1.00
  PPA(1)=1.23
  PF(1)=15.6
  QGAIN(1)=0.0
  OFFSET=0.0
  AOFF=0.0
  AINT=0.0
  OFFCUM=0.0
  TNUMB=8.20
C
C  WRITING THE HEADING TO FILE
C
  WRITE(1,*) 'CONTROL CONSTANTS TRIED'
  WRITE(1,*) 'PROPORTIONAL GAIN '
  WRITE(1,25) GAMMA
  WRITE(1,*) 'INTEGRAL GAIN '
  WRITE(1,25) GAMMA2
  WRITE(1,*) ' '
  WRITE(1,*) ' '
  WRITE(1,*) 'PERTURBATION VALUES'
  WRITE(1,*) ' '
  WRITE(1,*) 'CELL EQUATION'
  WRITE(1,25) ZPERTC
  WRITE(1,*) 'DISSOLVED FISHMEAL EQUATION'
  WRITE(1,25) ZPERTD
  WRITE(1,*) 'FISHMEAL EQUATION'
  WRITE(1,25) ZPERTF
  WRITE(1,*) 'NEUTRAL PROTEASE EQUATION'
  WRITE(1,25) ZPERTN
  WRITE(1,*) 'ALKALINE PROTEASE EQUATION'
  WRITE(1,25) ZPERTA
  WRITE(1,*) ' '
  WRITE(1,*) ' '
  WRITE(1,*) ' '
  WRITE(1,*) 'NUMB  QMOD  QPERT  OFFSET  DMOD  DPERT'
25  FORMAT(5X,F5.2)
C
C  THIS IS THE BEGINNING OF THE TIMESTEP LOOP
C
  DO 500 IK=1,206,1

```

```

C
C  EULER METHOD FOR THE MODEL
C
  FY(1) = X(IK)
  FY(2) = D(IK)
  FY(3) = PN(IK)
  FY(4) = PA(IK)
  FY(5) = F(IK)
C
  CELL = FY(1)
  DISV = FY(2)
  PNEU = FY(3)
  PALK = FY(4)
  FISH = FY(5)
C
C  SLOPES
C
  YD(1) = FMU(DISV)*CELL
  IF (TNUMB.GT.12) THEN
    YD(1) = FMUMON(DISV)*CELL
  END IF
  YD(2) = FFD(FISH,PNEU) - 1.0/YXD*FMU(DISV)*CELL
  IF (TNUMB.GT.12) THEN
    YD(2) = FFD(FISH,PNEU) - 1.0/YXD*FMUMON(DISV)*CELL
  END IF
  YD(3) = FNP(DISV)*CELL
  YD(4) = FAP(DISV)*CELL
  YD(5) = Q(IK) - FFD(FISH,PNEU)
C
C  UPDATED VALUES
C
  FY(1)=FY(1)+XTIME(IK)*YD(1)
  FY(2)=FY(2)+XTIME(IK)*YD(2)
  FY(3)=FY(3)+XTIME(IK)*YD(3)
  FY(4)=FY(4)+XTIME(IK)*YD(4)
  FY(5)=FY(5)+XTIME(IK)*YD(5)
C
C  STORING THE UPDATED VALUES
C
  X(IK+1)=FY(1)
  D(IK+1)=FY(2)
  PN(IK+1)=FY(3)
  PA(IK+1)=FY(4)
  F(IK+1)=FY(5)
C
C  EULER METHOD FOR THE PERTURBED MODEL
C
  PFY(1) = PX(IK)
  PFY(2) = PD(IK)
  PFY(3) = PPN(IK)
  PFY(4) = PPA(IK)

```

```

C      PFY(5) = PF(IK)
C
C      PCELL = PFY(1)
C      PDISV = PFY(2)
C      PPNEU = PFY(3)
C      PPALK = PFY(4)
C      PFISH = PFY(5)
C
C      SLOPES
C
C      PYD(1) = PFMU(PDISV)*PCELL
C      IF (TNUMB.GT.12) THEN
C          PYD(1) = PFMUMON(PDISV)*PCELL
C      END IF
C      PYD(2) = PFFD(PFISH,PPNEU) - 1.0/PYXD*PFMU(PDISV)*PCELL
C      IF (TNUMB.GT.12) THEN
C          PYD(2) = PFFD(PFISH,PPNEU) - 1.0/PYXD*PFMUMON(PDISV)*PCELL
C      END IF
C      PYD(3) = PFNP(PDISV)*PCELL
C      PYD(4) = PFAP(PDISV)*PCELL
C      PYD(5) = QGAIN(IK) - PFFD(PFISH,PPNEU)
C
C      UPDATED VALUES
C
C      PFY(1)=PFY(1)+XTIME(IK)*PYD(1)
C      PFY(2)=PFY(2)+XTIME(IK)*PYD(2)
C      PFY(3)=PFY(3)+XTIME(IK)*PYD(3)
C      PFY(4)=PFY(4)+XTIME(IK)*PYD(4)
C      PFY(5)=PFY(5)+XTIME(IK)*PYD(5)
C
C      STORING THE UPDATED VALUES
C
C      PX(IK+1)=PFY(1)
C      PD(IK+1)=PFY(2)
C      PPN(IK+1)=PFY(3)
C      PPA(IK+1)=PFY(4)
C      PF(IK+1)=PFY(5)
C
C      CHECK FOR NEGATIVE D
C
C      IF(D(IK+1).LT.0.0) D(IK+1)=0.0
C      IF(PD(IK+1).LT.0.0) PD(IK+1)=0.0
C
C      CALCULATING THE Q VALUE FOR THE CONTROLLED CASE
C
C      OFFSET=D(IK+1)-PD(IK+1)
C      OFF2(IK+1)=OFFSET
C      AOFF=ABS(OFFSET)
C      AINT=AINT+AOFF
C      OFFCUM=OFFCUM+OFFSET
C      DIFF(IK+1)=(OFF2(IK+1)-OFF2(IK))/XTIME(IK)

```

```

      PIGAIN=GAMMA**0.5*OFFSET+2**0.5*GAMMA2**0.75*OFFCUM+G3*DIFF(IK+1)
      QGAIN(IK+1)=Q(IK+1)+PIGAIN
C
C   CHECKING FOR NEGATIVE Q VALUE
C
      IF(QGAIN(IK+1).LT.0.0) QGAIN(IK+1)=0.0
C
C   CALCULATING THE NUMBER
C
      TNUMB=TNUMB+XTIME(IK)
C
C   WRITING THE RESULTS
C
      WRITE(5,114) TNUMB,Q(IK+1),QGAIN(IK+1),OFFSET,D(IK+1),PD(IK+1)
      WRITE(1,114) TNUMB,Q(IK+1),QGAIN(IK+1),OFFSET,D(IK+1),PD(IK+1)
114  FORMAT(F6.2,3X,F6.1,3X,F6.1,3X,F7.2,3X,F7.2,3X,F7.2)
C
C   END OF THE LOOP
C
500  CONTINUE
      WRITE(*,*) ' '
      WRITE(1,*) ' '
      WRITE(*,*) 'DEVIATION INDEX IS'
      WRITE(1,*) 'DEVIATION INDEX IS'
      WRITE(*,*) ' '
      WRITE(1,*) ' '
      WRITE(*,115) AINT
      WRITE(1,115) AINT
115  FORMAT(1X,F8.2)
      STOP
      END

```

BIOGRAPHICAL SKETCH

Max Kennedy was born and raised in Tauranga, New Zealand. He attended Tauranga Boys' College where he learned the basics of science. After gaining a scholarship he studied chemical and materials engineering at the University of Auckland. His interest in the life sciences was encouraged by Prof Peter Munro's course on biochemical engineering and the dairy industry.

During his stay at Auckland University Max had a number of summer jobs. His first job was at the Paeroa Dairy factory. He then became a fitters helper at a Tauranga construction firm. A short stay with the Ministry of Agriculture and Fisheries showed him how to measure the hardness of butter. The most interesting summer job was at the New Zealand Dairy Research Institute in Palmerston North where he learned about the difficulty in separating fines from dairy waste waters. His final summer job was at a margarine factory where he learnt about material balancing and quality control.

Max graduated top of his chemical and material engineering class and upon leaving university worked for the Industrial Processing Division of the Department of Scientific and Industrial Research (DSIR). It was at the DSIR that Max learnt first hand about biochemical engineering. Under the guidance of Don Bell Max's interest in fermentation grew. The DSIR encouraged Max to better himself by obtaining a PhD at an overseas university. This encouragement and financial support made Max's stay at M.I.T. possible.

Upon arriving at M.I.T. Max's life changed considerably. After learning the American language and forgetting lots of New Zealandisms Max settled into his studies. After a trip to Europe Max married his New Zealand girlfriend, Maria in Boston. Next to appear on the

scene was Emma, Max and Maria's daughter. Max's family then provided the motivation and encouragement to complete his thesis studies.

After graduation Max will return to New Zealand to continue working for the DSIR in biotechnology.